FEDERAL UNIVERSITY OF PARANÁ

ANA SILVIA PEDRAZZANI

USE OF ANAESTHETICS DURING HANDLING AND TRANSPORT OF CLOWN ANEMONEFISH *Amphiprion ocellaris*

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Thesis presented as partial requirement for the degree of Doctor of Veterinary Science of the Post-Graduation Course of Veterinary Science, Department of Agricultural Sciences, Federal University of Paraná.

Advisor: Prof. Dr. Antonio Ostrensky Neto

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A Comissão Examinadora da Defesa da Tese intitulada **"USO DE ANESTÉSICOS DURANTE O MANEJO E O TRANSPORTE DO PEIXE-PALHAÇO** *Amphiprion ocellaris*" apresentada pela Doutoranda **ANA SILVIA PEDRAZZANI** declara ante os méritos demonstrados pela Candidata, e de acordo com o Art. 79 da Resolução nº 65/09–CEPE/UFPR, que considerou a candidata APTA para receber o Título de Doutor em Ciências Veterinárias, na Área de Concentração em Ciências Veterinárias.

Curitiba, 26 de agosto de 2014

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I dedicate this work to my two loves, my daughter Beatriz, who always understood and was my battery charger when she gave me her intense hugs during all these years, and my partner Aldo, who lent his arms to endless reforms of the laboratory, his eyes when I couldn't see the problems solutions, and his ears to listen, always.

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My parents for all patience and efforts dedicated.

God for placing all these people in my way and for everything I have achieved so far.

The step of the ladder was not invented to rest, but just to support the foot, time necessary for man to put the other one a little higher.

Aldous Huxley

I prefer to be an ambulant metamorphosis than to have that old opinion formed on everything.

Raul Seixas

APRESENTAÇÃO

O trabalho denominado “Uso de anestésicos durante o manejo e o transporte do peixe-palhaço *Amphiprion ocellaris”* aborda o uso dos óleos essenciais de cravo, menta, e cânfora e dos compostos sintéticos propofol e MS-222 como agentes anestésicos para a espécie de peixe-palhaço *Amphiprion ocellaris*, com o propósito de avaliar a efetividade anestésica de cada composto, visando minimizar os impactos causados no seu grau de bem-estar durante os procedimentos de rotina do cultivo em cativeiro da espécie.

Esta tese é constituída por quatro capítulos principais, e outros dois capítulos constituem os apêndices I e II. O capitulo 1 intitulado “Anestesia através de imersão utilizando MS-222 e Propofol em peixes palhaços *Amphiprion ocellaris* (Cuvier, 1830)” e o capítulo 2 denominado “Efeito anestésico dos óleos de cânfora *Cinnamomum camphora* (L. J. Presl), cravo *Syzygium aromaticum* (L. Merrill & Perry) e menta *Mentha arvensis* (L.) em peixes palhaços *Amphiprion ocellaris* (Cuvier, 1830)” versam sobre a utilização de compostos sintéticos e óleos essenciais durante o manejo de *A. ocellaris*, respectivamente. Em ambos os capítulos, os tempos de indução e recuperação anestésicas são avaliados e as concentrações ideais de cada anestésico são estabelecidas. Os capítulos 3 “Efeito anestésico do Propofol e do MS-222 durante simulação de transporte de peixe-palhaço *Amphiprion ocellaris* e sua influência sobre a qualidade da água“ e 4 “Efeito do uso dos óleos essenciais de cravo, menta e cânfora sobre a qualidade da água de transporte de peixe palhaço *Amphiprion ocellaris* (Cuvier 1830)” avaliam os efeitos do uso dos anestésicos durante a simulação de transporte da espécie. São mensurados os principais parâmetros de qualidade de água e testadas diferentes períodos e densidades de transporte. O apêndice I discute as características de desempenho anestésico dos compostos estudados, e visa a divulgação científica em português do tema em questão. O apêndice II é um produto do estágio de doutorado realizado no Vero Beach Marine Laboratory, do Florida Institute of Technology, na Flórida, Estados Unidos, onde foi conduzido um estudo sobre comportamento reprodutivo, e desenvolvimento embrionário e larval inicial do red head goby *Elacatinus puncticulatus.*

ABSTRACT

The clown anemonefish *Amphiprion ocellaris* is the most popular fish species in aquarium trade. The culturing technology of this species has been enhanced as the demand increases. Consequently, there is a greater demand by consumers to obtain healthy animals. To ensure this quality, are required to be adopted mitigation measures of stress caused during the production process. The aim of this study was to evaluate the effectiveness of different anaesthetics during routine procedures as the biometrics and transporting of the clownfish *Amphiprion ocellaris*. Initially, two experiments were performed in which the fish were exposed individually for 15 consecutive minutes to five different concentrations of MS-222, propofol and clove, mint and camphor essential oils. The anaesthetic stages were verified for each fish (n =10/concentration). Posteriorly, the animals were transferred to containers containing clean water for observation of the time necessary to anaesthetic recovery. Other two experiments evaluated anaesthetic effects in confinement conditions similar to transport. Fish (n = 8/concentration/time) were submitted to three different concentrations in 6, 12 and 24 h of transport simulation. The main variables of water quality (dissolved oxygen, dissolved carbon dioxide, pH, total ammonia and gaseous ammonia were measured. Finally, were tested the effects of four different densities (5, 10, 15 and 20 fish L^{-1}) in transport simulation of *A. ocellaris* during 24h, under the effect of the same anaesthetics. Animals immersed in seawater containing the anaesthetics were packed in plastic packaging (n = 5 bags/density), in which was added pure oxygen in proportion 1: 2 (water/oxygen). Adopting the criteria of anaesthetic induction and recovery times and mortality of exposed animals, ideal concentrations of MS-222, propofol and clove, mint and camphor oils were defined respectively 80 mg L^{-1}, 0.7 mg $L^{-1,}$ 27 µl $L^{-1,}$ 70 µl L^{-1} and 500 µl L^{-1}. The use of 15 mg L^{-1} of MS-222 reduced metabolic waste disposal during 24h transport of species in densities between 10 and 15 fish L^{-1}. Propofol has provided no improvement in water quality of transport. The use of 25 µl L^{-1} of mint oil at maximum density of 10 fish L^{-1}, and clove and camphor oils at 5 µl L^{-1} and 120 µl L^{-1}, both at low density (5 fish L^{-1}), promoted a significant reduction of concentrations of total ammonia during *A. ocellaris* transport.

KEYWORDS: behavior, marine fish, ornamental, sedation, welfare.

RESUMO

O peixe-palhaço *Amphiprion ocellaris* é a espécie de peixe marinha ornamental mais comercializada ao redor do mundo. A tecnologia de cultivo desta espécie tem se aprimorado à medida que a sua demanda comercial aumenta. Consequentemente, tem havido um aumento do nível de exigência do mercado por animais cada vez mais saudáveis. Para assegurar esta qualidade é necessário que sejam adotadas medidas voltadas à mitigação do estresse causado durante o processo produtivo. O objetivo deste trabalho foi avaliar a efetividade de diferentes anestésicos durante procedimentos rotineiros, como a biometria e o transporte do peixe-palhaço *Amphiprion ocellaris*. Primeiramente, foram realizados dois experimentos nos quais os peixes (n=10/ concentração) foram expostos individualmente, por 15 minutos consecutivos, a cinco diferentes concentrações de MS-222, propofol e óleos de cravo, menta e de cânfora. Foram avaliados os estágios anestésicos atingidos por cada peixe em cada concentração testada. Posteriormente, os animais foram transferidos para recipientes contendo água limpa, para observação do tempo necessário para recuperação anestésica. Outros dois experimentos avaliaram os efeitos do uso dos anestésicos em condições de confinamento similares às de transporte. Os peixes (n=8/concentração/tempo) foram submetidos a três diferentes tempos simulados de transporte (6, 12 e 24h) e as principais variáveis de qualidade da água (oxigênio dissolvido, dióxido de carbono dissolvido, pH, amônia total e amônia gasosa) foram medidas. Por fim, foram testados os efeitos desses mesmos anestésicos para *A. ocellaris* em quatro densidades (5, 10, 15 e 20 px L^{-1}), em condições simuladas de transporte*,* durante período de 24h. Os animais foram expostos aos anestésicos através de imersão em água salgada contendo os anestésicos (diluídos ou dissolvidos, dependendo do caso). A seguir, os peixes e água foram transferidos para embalagens plásticas (n= 5 embalagens/densidade). Os sacos foram preenchidos com oxigênio puro na proporção 1:2 (água/oxigênio). Adotando-se como critérios o tempo de indução e de recuperação anestésica, além da mortalidade de animais expostos aos anestésicos, as concentrações ideais de MS-222, propofol e óleos de cravo, menta e cânfora foram definidas em, respectivamente, 80 mg L^{-1}, 0,7 mg L^{-1}, 27 µL L^{-1}, 70 µL L^{-1} e 500 µL L^{-1}. O uso de 15 mg L^{-1} de MS-222 reduziu a eliminação de resíduos metabólicos durante a simulação de transporte da espécie em densidades entre 10 e 20 px L^{-1}, em período de 24 h. O propofol não proporcionou efeitos positivos sobre a qualidade de água de transporte. A utilização de 25 µL L^{-1} de óleo de menta na densidade máxima de 10 px L^{-1}, e o óleos de cravo e cânfora nas concentrações de 5 µL e 120 µL L^{-1}, em baixa densidade (5 px L^{-1}) promoveram redução significativa das concentrações de N-AT (nitrogênio na forma de amônia total) durante o transporte de *A. ocellaris*.

PALAVRAS-CHAVE: bem-estar, comportamento, ornamental, peixe marinho, sedação.

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SUMMARY

CHAPTER 1. IMMERSION ANAESTHESIA USING MS-222 AND PROPOFOL IN THE CLOWNFISH *Amphiprion ocellaris* (CUVIER, 1830) [1]

ABSTRACT

The aim of this study was to evaluate the anaesthetic performances of propofol and MS-222 and establish their optimal concentrations as anaesthetics for *Amphiprion ocellaris* during handling in farming conditions. The following concentrations were evaluated: 50, 60, 70, 80 and 90 mg L^{-1} of MS-222; and 0.4, 0.5, 0.6, 0.7 and 0.8 mg L^{-1} of propofol. Ten fish were used per concentration. The animals were exposed to these concentrations for 15 minutes. During that period, the time required to achieve the different stages of anaesthesia were recorded. Next, the fish were placed in clean water to record the recovery period. All of the results were compared with those obtained from a corresponding control group in which the fish were subjected to the same handling procedure but were not exposed to any anaesthetic. The periods required for anaesthesia induction by propofol and MS-222 were similar. Primarily, considering the safety margin and the anaesthetic recovery period, it is recommended to use 80 mg L^{-1} of MS-222 to anaesthetise clownfish during handling for procedures such as biometrics and classification.

KEYWORDS: handling, ornamental, reef fish, sedation, welfare.

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Authors: A.S. Pedrazzani, R.G. Vilani and A. Ostrensky.

1.1 INTRODUCTION

The progress in veterinary medicine of fish, the enhancement of available technology in aquaculture, and the growing concern for farm animal welfare are responsible for the increased use of anaesthetics in aquaculture The use of chemicals to aid in physical restraint during fish handling improves both the handler and animal safety, since anaesthetics decrease the fish excitation and allow rapid procedures to be performed out of water (Sneddon, 2012).

To choose an appropriate anaesthetic should consider not only its effectiveness in fast fish immobilization but have the characteristics to promote a calm and safe recovery. In addition, it is recommended that the anaesthetic be nontoxic, easy to handle, water soluble and affordable (Ackerman *et al.*, 2013). Several products are used for immersion anaesthesia of fish, the most common being tricaine methane-sulphonate (MS-222), benzocaine, eugenol, metomidate, 2-phenoxyethanol, and quinaldine (Neiffer e Stamper, 2009).

MS-222, a compound derived from benzocaine is easily dissolved in water, absorbed through the gills, and processed in the liver and kidneys and discharged through the gills, urine and bile (Neiffer e Stamper, 2009). However, it may cause hypoventilation and damage to the fish retina and even to humans during handling (Sladsky *et al.*, 2001). Additionally, it is known that it is not effective for all fish species (Fleming *et al.*, 2003). In Brazil, this anaesthetic is not easily acquired, besides being relatively high in cost compared to other anaesthetics commonly used in fish farming (Roubach e Gomes, 2001).

Propofol (2,6 diisopropyl phenol) is an anaesthetic of rapid metabolization, has no cumulative effect, and allows a brief anaesthetic recovery (Gholipourkanani e Ahadizadeh, 2013). In mammals, it is used intravenously, and for fish this route was evaluated for spotted bamboo shark (*Chiloscyllium plagiosum*) (Miller *et al.*, 2005) and sturgeon *Acipenser oxyrinchus* (Fleming *et al.*, 2003). Alternatively, propofol can be used for fish anaesthesia by immersion. The application by water dilution has already been studied in goldfish (*Carassius auratus*) (Gholipourkanani e Ahadizadeh, 2013) and in jundiá *Rhamdia quelen* (Gressler *et al.*, 2012). Nevertheless, there were no studies on immersion anaesthesia using propofol for marine fishes.

The clown anemonefish (*Amphiprion ocellaris*) has great economic importance as an ornamental species, being widely used in marine aquariums, which has contributed to popularize and spread its cultivation in captivity (Madhu *et al.*, 2006; Kodama *et al.*, 2011). The aim of this study was to comparatively evaluate the anaesthetic performances of propofol and MS 222 and determine ideal concentrations for *A. ocellaris* anaesthesia during handling.

1.2 MATERIAL AND METHODS

1.2.1 ACQUISITION AND MAINTENANCE OF ANIMALS

Two hundred juvenile *Amphiprion ocellaris*, with a total length of 2.75 cm ± 0.39 cm (mean/standard deviation) and weight of 0.47 g ± 0.42 g (mean/standard deviation), were acquired from Azul Fish Farm (São Paulo, Brazil) and transported in plastic bags, containing water and pure oxygen in a 1:2 ratio, at a density of 20 fish L^{-1} of water. In the laboratory, the animals underwent a process of gradual acclimation to temperature, pH and salinity. Animals were transferred to glass maintenance tanks measuring 100 x 40 x 50 cm (length x width x height), with a black plastic cover on the back to reduce interference from external light. The tanks were interconnected by a saltwater recirculation system. Fish were kept in these tanks for 10 days. Salinity, temperature and pH were kept at 30, 25 °C, and 7.9-8.1, respectively. Partial water changes of 25% of the volume of the tanks were performed weekly. The concentration of total ammonia (TA) was measured every three days and was always kept at levels below 0.25 mg L^{-1} of total ammonia nitrogen (TA-N).

The fish were fed twice daily *ad libitum* with a commercial pellet feed containing 47.5% crude protein. Food debris and faecal matter present in the bottom of the tanks were removed by siphoning one hour after feeding.

1.2.2 DETERMINATION OF THE CONCENTRATIONS USED

Stock solutions were initially prepared for each product tested. Propofol was diluted in distilled water at a 1:10 ratio. MS-222 was dissolved at a ratio of 5 g of product per litre of distilled water. The concentrations of anaesthetics used were determined in two steps. Initially, a pilot experiment was performed to test the anaesthetic effect of the initial concentrations, based on literature, of 0.2 mg L^{-1} of propofol (2,6 diisopropylphenol, Provine 1%, Meizler®) and 20 mg L^{-1} of MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich®), which were diluted and dissolved in water, respectively. Each animal was individually exposed to an anaesthetic concentration in beakers containing water with a salinity of 30 g L^{-1} for a predetermined period of 15 minutes. After the exposure period, the response displayed by the animal to the respective anaesthetic was evaluated, and the animal was transferred to a tank with clean water. Then, depending on the result obtained, a new concentration, increasing 0.1 mg L^{-1} of propofol or 10 mg L^{-1} of MS-222, were tested with a new fish being subjected to the anaesthetic. This procedure was repeated successively until the tested dose was sufficient to produce the anaesthetic stage V (TABLE 1) or delayed recovery (greater than 40 minutes). Using these criteria, the maximum concentrations tested were 0.9 mg L^{-1} for propofol and 100 mg L^{-1} for MS-222. Thus, the pre-tests were performed using the minimum number of animals necessary to assess the responses to the anaesthetics and minimising the risk of death for the fish tested.

In the second stage (n = 110), the last five tested concentrations before the maximum one, for each anaesthetic, were used to the definitive experiment. This consisted of a completely randomised factorial study in which five different concentrations of each product (n = 10 fish/concentration/anaesthetic) were evaluated. MS-222 was tested individually at the concentrations of 50; 60; 70; 80 and 90 mg L^{-1}, and propofol was evaluated at the concentrations of 0.4; 0.5; 0.6; 0.7 and 0.8 mg L^{-1}. All of the results were compared with those obtained in the corresponding control group (n = 10), in which the fish were subjected to the same handling procedure but were just exposed to clean water.

TABLE 1. Anaesthetic stages in fish and behavioural characteristics for each stage.

Anesthesia stage	Behavioral parameters
I	Absence of reaction to touch and to visual stimulus.
II	Initial loss of balance, characterized by difficulty to maintain normal swimming position, alternating normal and irregular (lateral) swimming.
III	Total loss of balance, uncoordinated swimming.
IV	Minimal opercular movement, no swimming.
V	No opercular beating.

SOURCE: ADAPTED FROM ROSS & ROSS (2008).

To evaluate the anaesthetic effects, fish were randomly collected from a maintenance tank and individually transferred to glass beakers containing 1 L of saltwater with a salinity of 30 g L^{-1} and a specific dose of the product for each experimental treatment. The total exposure time for each animal was 15 minutes. During that period, the times required for the fish to reach each stage of anaesthesia (induction periods) were monitored and recorded. During anaesthesia, the animals were subjected to biometrics. The weight was measured using a precision scale (AY 220, Shimadzu, Brazil), and the length was measured using a manual calliper (Vonder, Brazil).

After the individual exposure period, the animals were transferred separately to other containers similar to those used to induce anaesthesia that contained 1 L of saltwater free of any residual anaesthetic. The time required to return to the baseline condition was measured and recorded. Animals were considered recovered when they returned to the vertical position and normal swimming. Fish were also monitored for mortality and feeding behaviour twice daily during the 72 hours following exposure to the drugs. When providing feed, the search and capture of food were observed for five minutes.

1.2.3 STATISTICAL ANALYSIS

The results obtained using each anaesthetic were analysed separately, and the anaesthetic performance of the two products were subsequently compared, both with respect to the induction time and recovery from anaesthesia. The data normality was previously assessed by the Shapiro-Wilk test. Because the data did not fit the normal (Gaussian) curve, statistical differences between the induction and recovery times were assessed by the Mann-Whitney and Kruskal-Wallis tests ($p<0.05$). All of the analyses were performed using the Statsoft Statistica^{TM} software version 10.0.

1.3 RESULTS

As expected, the control group did not show any characteristic signs of anaesthesia, and mortality was not observed in this treatment. The concentrations of 70, 80 and 90 mg L^{-1} of MS-222 induced stage IV of anaesthesia, and the greatest dose induced stage V (medullary collapse) in two animals (TABLE 2). From the five propofol concentrations assessed, only the concentrations of 0.7 and 0.8 mg L^{-1} induced stage IV in all of the animals evaluated. Stage V was not induced in any of the fish exposed to the tested concentrations of propofol.

TABLE 2. NUMBER OF *Amphiprion ocellaris* THAT REACHED EACH ANAESTHETIC STAGES AFTER 15 MINUTES OF EXPOSURE AND THEIR RESPECTIVE CONCENTRATION, AND OBSERVED MORTALITY FOR THE FIRST 24 HOURS AFTER ANAESTHETIC INDUCTION.

Anaesthetic		**STAGE**					
	mg L^{-1}	**I***	**II****	**III*º**	**IVº**	**Vºº**	**MORTALITY**
MS-222	50	10	10	05	03	00	00
	60	10	10	07	05	00	00
	70	10	10	10	10	00	00
	80	10	10	10	10	00	00
	90	10	10	10	10	02	02
PROPOFOL	0.4	10	10	10	00	00	00
	0.5	10	10	10	05	00	00
	0.6	10	10	10	07	00	00
	0.7	10	10	10	10	00	00
	0.8	10	10	10	10	00	00

KEY: * ABSENCE OF REACTION TO EXTERNAL STIMULATION, ** PARTIAL BALANCE LOSS, *º TOTAL BALANCE LOSS, º OPERCULAR BEATING REDUCTION, ºº NO OPERCULAR BEATING.

There was a moderate negative correlation between the anaesthetic concentration and the induction time for fish to reach anaesthetic stage IV, and there was a weak positive correlation between the concentration and anaesthetic recovery time for animals exposed to MS-222. There was no correlation between these variables in the case of propofol (TABLE 3).

TABLE 3. RESULTS OF THE ANALYSIS OF THE LINEAR CORRELATION OBTAINED BETWEEN THE EFFECTIVE CONCENTRATIONS OF PROPOFOL AND MS-222 AND THE INDUCTION TO ANAESTHETIC STAGE IV AND ANAESTHETIC RECOVERY TIME IN *Amphirion ocellaris.*

Variable	Treatment	Tendency	r^2	P	Correlation
Induction (stage IV)	MS-222	-	0,58	0,00*	Moderate
	Propofol	+	0,02	0,40	NS
Recovery	MS-222	+	0,31	0,00*	Weak
	Propofol	-	0,04	0,14	NS

NOTE: COEFFICIENT OF DETERMINATION (r^2) BETWEEN 0-0.19: VERY WEAK CORRELATION; 0.20-0.39: WEAK; 0.40-0.69: MODERATE; 0.70-0.89: STRONG; ABOVE 0.89: VERY STRONG. * SIGNIFICANT CORRELATIONS (P<0.05). NS: NOT SIGNIFICANT.

The times required for the induction of stages I, II and III did not differ between the two propofol concentrations (FIGURE 1). However, the period required to reach stage IV was longer when using the concentration of 0.8 mg L^{-1} (708.5 seconds) compared to that of 0.7 mg L^{-1} (280 seconds) ($p=0.0009$).

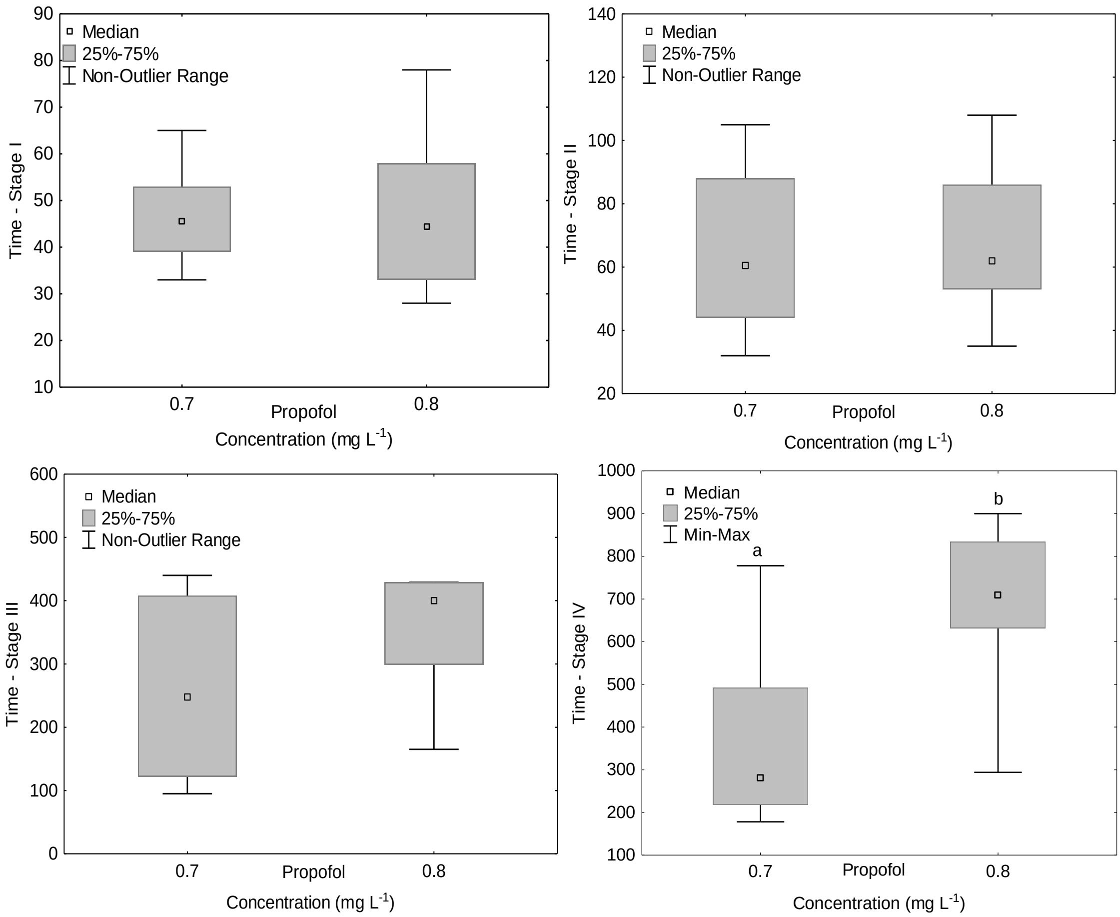

FIGURE 1. TIME (SECONDS) REQUIRED TO INDUCE ANAESTHESIA IN *Amphiprion ocellaris* USING PROPOFOL. DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES BETWEEN THE CONCENTRATIONS (P<0.05).

The concentrations of 70 and 80 mg L-1 of MS-222 induced anaesthetic stages I and II at similar times ($p > 0.05$); however, the periods required to induce stages III and IV were longer for the lower (medians 350.5 and 494 seconds, respectively) MS -222 concentration than for the higher (medians 105 and 353.5 seconds) concentration (FIGURE 2). When analysed together, the times required to reach stage IV in *Amphiprion ocellaris* were 577 and 418 seconds for induction by exposure to propofol and MS-222, respectively.

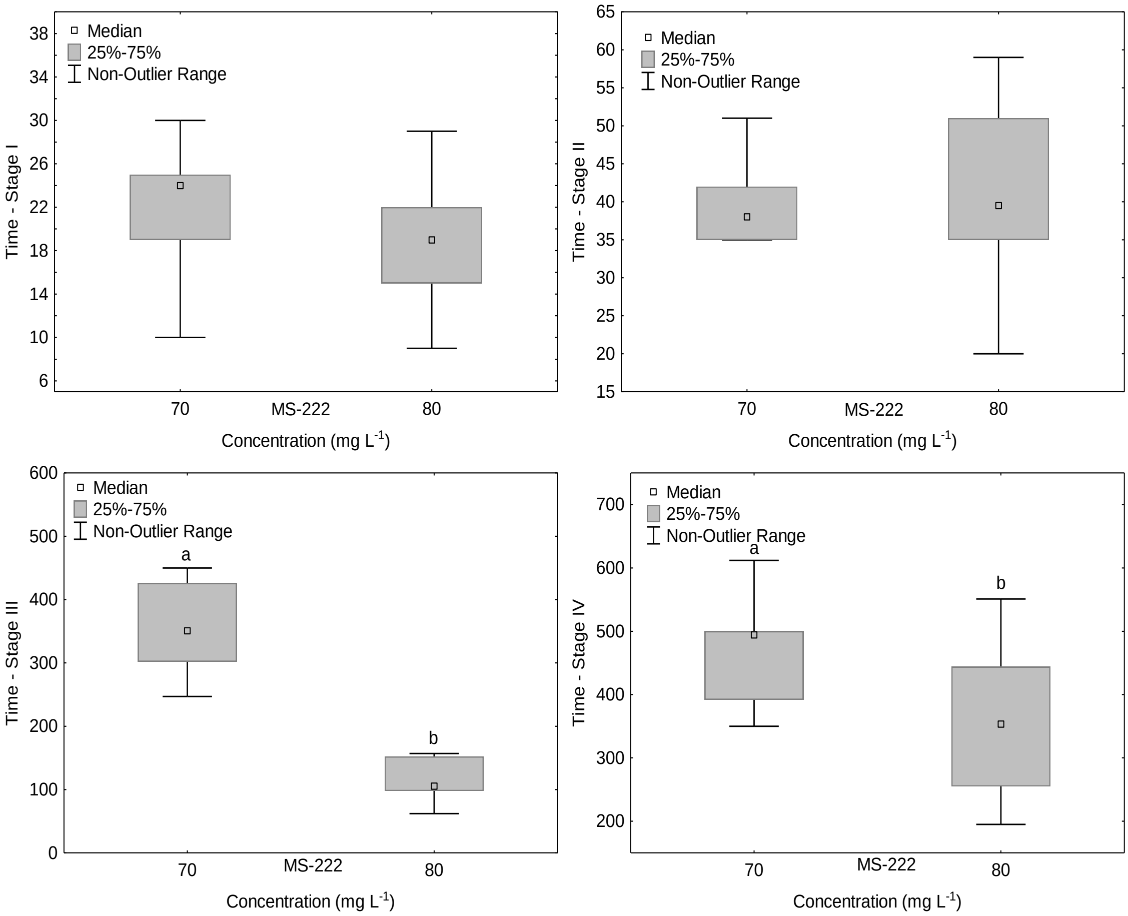

FIGURE 2. TIME (SECONDS) REQUIRED TO INDUCE ANAESTHESIA IN *Amphiprion ocellaris* USING MS-222. DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES BETWEEN THE CONCENTRATIONS (P<0.05).

There was no significant difference between the recovery periods of fish subjected to concentrations of 0.7 and 0.8 mg L^{-1} of propofol, which were 1,507.5 and 1,415 seconds, respectively (FIGURE 3). A similar pattern was observed when comparing the MS-222 concentrations of 70 and 80 mg L^{-1}, for which the animals exhibited similar recovery times (203.5 and 255 seconds, respectively). No changes in feeding behaviour were observed, and there was no mortality after exposure to both anaesthetics at the concentrations tested.

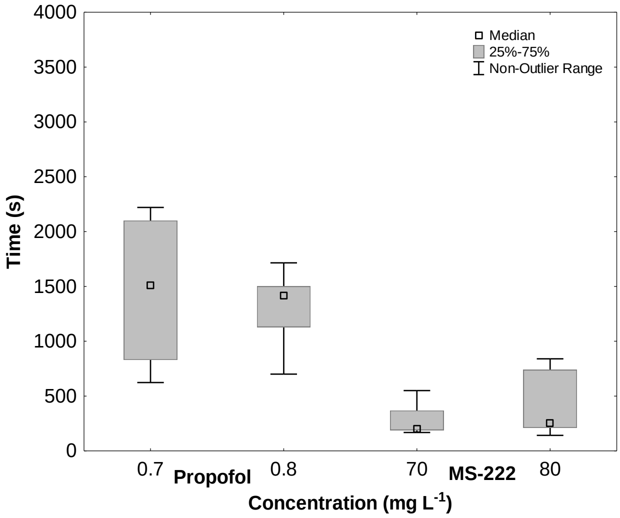

FIGURE 3. TIME (SECONDS) REQUIRED FOR RECOVERY FROM ANAESTHESIA IN *Amphiprion ocellaris* AFTER EXPOSURE TO PROPOFOL AND MS-222.THERE WERE NO SIGNIFICANT DIFFERENCES BETWEEN THE VARIOUS TESTED CONCENTRATIONS OF THE SAME PRODUCT.

1.4 DISCUSSION

Many biological and environmental factors can affect the anaesthetic process in fish. Generally, the rate of anaesthetic efficacy is derived from the relationship between the gill area and the body weight of the animal, which varies according to the species (Coyle *et al.*, 2004). Furthermore, the metabolism of the fish affects the absorption and consequent anaesthetic induction, and finally, the degree of ionisation of the anaesthetic agent and its solubility in fat also make its effects species-specific (Keene *et al.*, 1998).

In the present study, both of the synthetic compounds evaluated have anaesthetic effects in *A. ocellaris*. The concentrations of 70 and 80 mg L^{-1} for MS-222 and 0.7 and 0.8 mg L^{-1} for propofol were considered adequate because they induced anaesthetic stage IV in all of the exposed animals without leading to medullary collapse and consequently death. Thus, only these concentrations were compared in terms of induction times (stages I to IV) and recovery from anaesthesia.

From the two analysed MS-222 concentrations, 80 mg L^{-1} had the fastest effect in this species, making it possible to reach stage IV in 373.5 s (median). This concentration is lower than that found for the seahorse *Hippocampus kuda*, which was 125 mg L^{-1} (Pawar *et al.*, 2011), and the guppy *P. reticulata*, which was 125 to 200 mg L^{-1} (Chambel *et al.*, 2013). However, this concentration comes very close to concentrations suggested for other ornamental fish, such as the zebrafish *Danio rerio* (between 75 and 125 mg L^{-1}) and the red discus *Symphysodon discus* (between 75 and 100 mg L^{-1}) (Chambel *et al.*, 2013).

The propofol concentration of 0.7 mg L^{-1} induced the first four anaesthetic stages safely and more quickly (280 s) compared to the remaining concentrations. This dose is equivalent to only 10% of that used for anaesthesia in goldfish *C. auratus* (7 mg L^{-1}) (Gholipourkanani e Ahadizadeh, 2013) and is much lower than the dose required to anesthetise the catfish *R. quelen* (5 to 12 mg L^{-1}) (Gressler *et al.*, 2012), which in such cases may be related to the larger size of the organisms used. Additionally, the time of 460 seconds required to induce stage IV anaesthesia in goldfish was shorter than the induction time found for the clownfish, which was 577 seconds.

According to Gholipourkanani e Ahadizadeh (2013), goldfish exhibited recovery periods from propofol anaesthesia of 532 s, closed to double the five minutes recommended as an appropriate upper limit for return from anaesthesia (Ross e Ross, 2008). Clownfish, in turn, took approximately five times longer than this optimal time to recover from anaesthesia, which may be related to the fact that propofol causes cardiovascular depression, thereby delaying the elimination of the anaesthetic by the body (Fleming *et al.*, 2003).

In contrast, MS-222 allowed a rapid (235.5 s) recovery of the clownfish, likely due to its feature of accelerating heart and respiratory rates (Keene *et al.*, 1998), thus allowing the compound to be excreted more rapidly from the body of the fish. MS-222 also yielded a faster recovery than the oil of the clove *Eugenia caryophyllata*, which has been used as an anaesthetic for the rainbow trout *Oncorhynchus mykiss* (Keene *et al.*, 1998), red-bellied pacu *Piaractus brachypomus* (Sladsky *et al.*, 2001) and clownfish *A. ocellaris* (Pedrazzani e Ostrensky, 2014).

When using anaesthetics, it is expected that there will be a strong negative correlation between the applied concentration and the time required to induce anaesthesia to the desired stage, as observed previously for several fish species (Weber *et al.*, 2009; Pramod *et al.*, 2010; Ibarra-Zatarain *et al.*, 2011; Pawar *et al.*, 2011). However, in the present study, this correlation was not observed for propofol and was only moderate in tests with MS-222. This result is likely related to the relatively short interval between the effective concentrations and those unsuitable for the anaesthesia of *A. ocellaris*. Namely, for this species, there are narrow margins separating the concentrations of MS222 and propofol required to reach anaesthetic stage IV, from concentrations that lead only to lower anaesthetic stages to those that that can cause medullary collapse and the death of the fish. This narrow safety margin may hinder the use of both anaesthetics during handling, requiring more rigorous preparation and application of these products. However, at the recommended concentrations, both of the products evaluated provided satisfactory anaesthetic performance in *A. ocellaris* regarding the possibility of obtaining stage IV anaesthesia and the lack of mortality after anaesthetic exposure or changes in food intake after the recovery period.

Summarizing, propofol (0.7 to 0.8 mg L^{-1}) and MS-222 (70 to 80 mg L^{-1}) are able to induce adequate anaesthetic deep to handling in clownfish, and are similar in anaesthetic induction time, stage of anaesthesia and safety. However, the anaesthetic recovery period is shorter using MS-222.

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CHAPTER 2. THE ANAESTHETIC EFFECT OF CAMPHOR (*Cinnamomum camphora),* CLOVE (*Syzygium aromaticum*) AND MINT (*Mentha arvensis*) ESSENTIAL OILS ON CLOWN ANEMONEFISH, *Amphiprion ocellaris* (CUVIER 1830) [2]

ABSTRACT

The aim of this study was to evaluate the use of clove (*Syzygium aromaticum*), camphor (*Cinnamomum camphora*) and mint (*Mentha arvensis)* essential oils as anaesthetics during the management of clown anemonefish (*Amphiprion ocellaris)*. For 15 min, the animals were subjected to concentrations of 5, 10, 20, 27 and 35 µL L^{-1} of clove oil, 17, 35, 50, 70 and 100 µL L^{-1} of mint oil, and 200, 400, 500, 550 and 600 µL L^{-1} of camphor oil (tested in 10 animals per concentration). A control group (without anaesthetic) and a complementary group, which was exposed to ethanol, were also evaluated. After exposure to the anaesthetic, the fish were transferred to clean water to assess recovery. All of the essential oils produced an anaesthetic effect on *A. ocellaris*. The 27, 70 and 500 µL L^{-1} concentrations of clove, mint, and camphor oils promoted surgical anaesthesia after 310.5, 312.0, and 535.0 s (medians), respectively. The recovery times of fish exposed to these same concentrations were 396, 329.5 and 229 s, respectively. The decision of which oil to use is dependent on the management situation and the consideration of the induction and recovery times of each essential oil.

KEYWORDS: handling, ornamental, reef fish, sedation, welfare.

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2.1 INTRODUCTION

Globally, it is estimated that at least one hundred different species of marine fish are produced by breeders for ornamental proposes (Wittenrich, 2007). According to the Global Marine Aquarium Database (Green, 2008), the most exported marine species between 1997 and 2002 was the clown anemonefish (*Amphiprion ocellaris)*. This species alone was responsible for 25% of the total amount of ornamental marine fish trade worldwide (Wabnitz *et al.*, 2003) and is one of the five most imported species by the United States of America (Rhyne *et al.*, 2012). The *A. ocellaris* presents several favourable characteristics that make it exceptionally well suited for rearing in captivity, such as its known ability to reproduce effectively, a high market value and easy adaptation to captivity conditions (Wittenrich, 2007; Kodama *et al.*, 2011).

However, activities associated with management during culturing and preparation for trade, such as catching and classifying individuals by size, are stressful (Pedrazzani *et al.*, 2007). These handling actions may result in negative behavioural and physiological effects, such as a decrease in feeding, enhancement of aggressive behaviour and susceptibility to disease (Ross e Ross, 2008). The use of anaesthetics during rearing procedures is an alternative to minimise these deleterious effects. Although anaesthesia has been used to immobilise fish, it can also be utilised to reduce physical damage incurred during handling (Vidal *et al.*, 2007) and the associated mortality and morbidity (Cooke *et al.*, 2004).

Many synthetic anaesthetics have been used on fish, and the most common are MS-222 (tricaine methane sulphonate), quinaldine and benzocaine (methyl-p-aminobenzoate) (Ross e Ross, 2008; Neiffer e Stamper, 2009). However, these anaesthetics are expensive or may be difficult to acquire (Roubach *et al.*, 2005). Because of these complications, the use of vegetal essential oils has emerged as an alternative option for fish anaesthesia (Readman *et al.*, 2013). Essential oils are obtained from plants, such as clove (*Syzygium aromaticum)*, mint (*Mentha sp.)*, and bushy matgrass (*Lippia alba)*. These oils have been recommended for fish anaesthesia due to their low costs, easy accessibility, efficacy, and environmental safety (Iversen *et al.*, 2003; Cunha *et al.*, 2011).

Several studies have shown the anaesthetic effectiveness of clove oil, which is composed of 70-90% eugenol, when used in freshwater (Inoue *et al.*, 2003; Vidal *et al.*, 2007; Oliveira *et al.*, 2009; Simões e Gomes, 2009) and marine fish that are intended for human consumption (Souza *et al.*, 2012) as well as in marine (Cunha e Rosa, 2006) and freshwater ornamental species (Bittencourt *et al.*, 2012). Similarly, some recent studies have demonstrated the effectiveness of mint oil and its main component, menthol, for freshwater (Gonçalves *et al.*, 2008; Oliveira *et al.*, 2009; Pádua *et al.*, 2010; Teixeira *et al.*, 2011; Mello *et al.*, 2012) and marine fish anaesthesia (Souza *et al.*, 2012).

White camphor (*Cinnamomum camphora)* has been used to treat inflammatory diseases, such as rheumatism, bronchitis, asthma, indigestion, and muscular pain in humans. It has also been used as a local anaesthetic for humans (Lee *et al.*, 2006). Some studies have been performed to examine the immunological effects of stout camphor (*Cinnamomum kanehirae)* essential oil on white shrimp (*Litopenaeus vannamei)* (Yeh *et al.*, 2009), but there are no studies that have evaluated the anaesthetic effects of these plant compounds on aquatic animals. The purpose of this study was to evaluate the anaesthetic effects of camphor (*C. camphora)*, clove (*S. aromaticum)* and mint (*Mentha arvensis)* essential oils and to determine the ideal concentration of each essential oil necessary to safely anaesthetise *A. ocellaris* juveniles during the handling process.

2.2 MATERIALS AND METHODS

The experiments were performed in the Research Laboratory of Aquatic Organisms (LAPOA) of the Integrated Group for Studies in Aquaculture (GIA), Federal University of Paraná (UFPR), Curitiba, Parana, Brazil.

2.2.1 ANIMAL ACQUISITION AND HOLDING

Two hundred *A. ocellaris* juveniles (0.48 ± 0.21 g, 2.59 ± 0.61 cm; mean ± SD) were acquired from the Azul Fish Farm, São Paulo, Brazil, in February 2013. They were transported in plastic bags that contained water and pure oxygen in a 1: 2 ratio. Each bag held 10 fish L^{-1}. The bags were shipped in isothermal boxes that were transported by air for seven hours.

In the laboratory, the bags containing the fish were gradually acclimatised over thirty min to avoid differences in water temperature, pH, and salinity. Then, the fish were divided and transferred into three glass maintenance tanks (100 cm length × 40 cm width × 50 cm depth) with the back painted black to reduce the effects of incident external light, where were kept for 10 days prior the experiment. The fish were fed *ad libitum* twice daily with granulate commercial feed containing 47.5% crude protein (Tetra, Germany). The remaining food and faeces were siphoned out of the tank one hour after feeding.

2.2.2 MAINTENANCE OF WATER QUALITY

The tanks were interconnected in a saltwater recirculation system with a protein skimmer and biological filtration as well as a water heater and cooler. The water quality parameters during the experiment were maintained at salinity of 30 ± 0.45 g L^{-1}, temperature 25 ± 0.46 ºC and pH 7.9 ± 0.4 (mean ±SD). The total ammonia-N was measured every three days and never exceeded 0.25 mg L^{-1} or the equivalent of 0.06 mg L^{-1} of non-ionised ammonia-N ($N\text{-}NH_3$). Water changes were performed weekly by removing 25% of the tank volume and replacing it with clean, properly conditioned water.

2.2.3 ANAESTHETIC AGENTS

The chemical composition of camphor, clove, and mint essential oils were provided by the chemical manufacturer Ferquima Indústria e Comércio LTDA™ (2013) (TABLE 4).

TABLE 4. CHEMICAL COMPOSITION (%) AND DENSITIES (G/CM³) OF CAMPHOR, CLOVE, AND MINT ESSENTIAL OILS.

ESSENTIAL OIL	MAIN COMPONENTS	DENSITY
Camphor	35.5 1.8-cineole; 30.0 limonene; 13.0 alpha-pinene; 10.0 para-cymene.	0.88
Clove	85.0 eugenol; 13.0 beta caryophyllene; 0.16 alpha copaene + methyl eugenol.	1.04
Mint	37.0 l-menthol; 20.25 menthone; 6.75 limonene; 7.48 isomenthone; 4.60 menthyl acetate; 1.81 isopulegone; 1:39 pulegone; 0.08 carvone; 0.34 cineole.	0.90

SOURCE: FERQUIMA INDÚSTRIA E COMÉRCIO LTDA.

2.2.4 ANAESTHETIC INDUCTION

To assess the ideal anaesthetic concentration, a pilot trial was performed for all of the substances at 5 $\mu L\ L^{-1}$ (minimum concentration possible to measure). After 15 min of exposure, the fish anaesthesia characteristic behaviour (TABLE 5) was evaluated. Additionally, several concentrations were tested to induce the desired level of anaesthesia (stage IV).

TABLE 5. ANAESTHETIC STAGES IN FISH AND CHARACTERISTIC BEHAVIOR AT EACH STAGE.

Anaesthetic stage	Behavior parameters
I – sedation	Loss of reaction to touch and visual perception.
II – light anesthesia	Loss of balance and normal natatory motion interchanged with irregular lateral swimming.
III – deep anesthesia	Total loss of balance, uncoordinated swimming.
IV – surgical anesthesia	Reduction of opercular beatment, absence of natatory motion.
V – medullary colapse	Absence of opercular beat, death.

SOURCE: ADAPTED FROM ROSS E ROSS (2008).

Once the upper and lower limits for each anaesthetic were established, a randomised factorial study was executed for five concentrations of the three essential oils tested. The final anaesthetic concentrations evaluated were 5, 10, 20, 27 and 35 µL L^{-1} of clove, 17, 35, 50, 70 and 100 µL L^{-1} of mint, and 200, 400, 500, 550 and 600 µL L^{-1} of camphor oils. Stock solutions were prepared by dilution at a 1: 10 ratio of oil to ethanol (100 g L^{-1} of 100% ethanol). The composition of these stock solutions made it necessary to test the anaesthetic effect of the oil diluent (ethanol) at the maximum concentration used in the dilution processes (35, 100 and 600 µL L^{-1}). The results were compared to a control group in which fish were submitted to the same procedures but not exposed to any anaesthetic.

For anaesthetic induction, 10 fish per concentration were randomly collected from the tanks and transferred separately into glass beakers containing one litre of saltwater with the established oil concentration, where they were maintained individually for 15 min. During this period, the time required to reach each anaesthetic stage was monitored and recorded (induction period). After the required time and while they were still under anaesthesia, biometric measurements were performed. The weight was obtained using a precision scale (AY 220, Shimadzu, Brazil) and the length was obtained using a pachymeter (Vonder, Brazil).

Five other tanks with one litre of clean water were used to evaluate the recovery time of the fish exposed to all treatments. The animals that returned to the vertical position and were able to swim were considered recovered. Finally, feeding behaviour and mortality were measured and recorded twice daily for five min during the 72 h following oil exposure.

2.2.5 STATISTICAL ANALYSIS

Data obtained from each treatment were analysed separately, and the anaesthetic performances of all essential oils were compared. The data normality was evaluated using the Shapiro-Wilk test. The data did not fit into a Gaussian curve; therefore, the anaesthetic induction and recovery times were analysed for significant differences using the Kruskal-Wallis test ($p<0.05$) followed by multiple comparisons of the mean ranks. Finally, a linear regression analysis was used to evaluate the relation between the biometric parameters of the fish and the observed anaesthetic stages and recovery period (CI 95%). The statistical package used for analysis was Statistica Statsoft^{TM} (V. 10.0).

2.3 RESULTS

No anaesthetic effects of fish mortality were exhibited by the control or ethanol-treated groups. Fish exposed to all concentrations of clove, mint and camphor oils reached stages I and II. Stages III and IV were only achieved by the elevated concentrations of each product. Only fish exposed to the 35 $\mu L\ L^{-1}$ concentration of clove oil registered stage V (medullary collapse) (TABLE 6). At this concentration, four fish died during exposure or within 24 h following exposure.

TABLE 6. NUMBER OF FISH THAT REACHED ANAESTHETIC STAGES UNDER EFFECT OF RESPECTIVE CONCENTRATIONS (µL L-1) OF CLOVE, MINT AND CAMPHOR ESSENTIAL OILS. ALSO MORTALITY OCCURRED DURING THE 24 HOUR PERIOD IMMEDIATELY FOLLOWING ANAESTHETIC INDUCTION.

OIL	**CONCENTRATION**	**STAGE**					
		I*	**II****	**III*º**	**IVº**	**Vºº**	**MORTALITY**
Clove	5	10	10	0	0	0	0
	10	10	10	10	5	0	0
	20	10	10	10	10	0	0
	27	10	10	10	10	0	0
	35	10	10	10	10	2	6
Mint	17	0	0	0	0	0	0
	35	10	10	0	0	0	0
	50	10	10	10	10	0	0
	70	10	10	10	10	0	0
	100	10	10	10	10	0	4
Camphor	200	10	10	0	0	0	0
	400	10	10	6	3	0	0
	500	10	10	7	5	0	0
	550	10	10	10	7	0	0
	600	10	10	10	9	0	0

* SEDATION, ** LIGHT ANESTHESIA, *º DEEP ANESTHESIA, º SURGICAL ANESTHESIA, ººMEDULLARY COLAPSE.

The induction and recovery times of the concentrations that were induced until anaesthetic stage IV in the majority of fish were compared (Figures 4 and 5). The 20 µL L^{-1} clove oil concentration promoted slower induction to stages I, II and III than did the 35 µL L^{-1} concentration (n= 10; p= 0.035; 0.040; and 0.013, respectively), even though both provoked surgical anaesthesia (stage IV) in a similar period. The 100 mL L^{-1} concentration of mint oil promoted all anaesthetic stages in *A. ocellaris* at a faster rate than the 50 µL L^{-1} concentration (n= 10 per concentration; p = 0.00). Stages I, II and IV were also induced more quickly when a higher dosage was used compared to an intermediate dosage (70 µL L^{-1}) (in all stages p = 0.00). The three highest camphor oil concentrations (500, 550 and 600 µL L^{-1}) did not present significant differences in the induction time of the anaesthetic stages (n=10 per concentration, p>0.05). In addition, fish agitation was observed during the first moments of exposure to camphor oil. The 27, 70 and 500 µL L^{-1} concentrations of clove, mint, and camphor oils promoted surgical anaesthesia in 310.5, 312.0 and 535.0 s (medians), respectively.

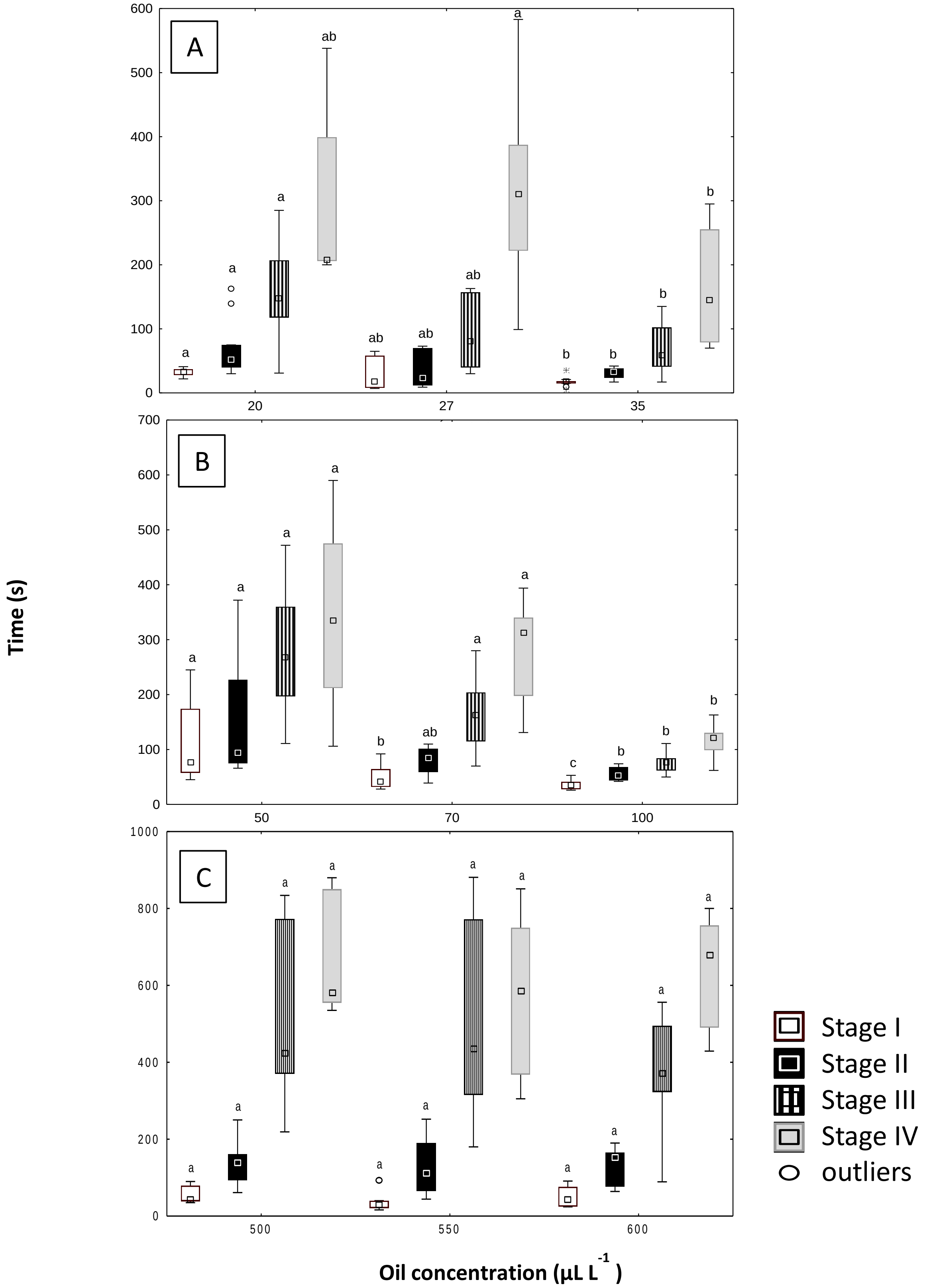

FIGURE 4. TIME (SECONDS) FOR ANAESTHETIC INDUCTION OF THE FIRST FOUR STAGES IN A. OCELLARIS THROUGH THE USE OF CLOVE (A), MINT (B) AND CAMPHOR OILS (C). STAGE I: LOSS OF RESPONSE TO EXTERNAL STIMULI, II: PARTIAL LOSS OF BALANCE; III: COMPLETE LOSS OF BALANCE; IV: REDUCTION OF OPERCULAR BEATING. DIFFERENT LETTERS INDICATE STATISTICAL DIFFERENCE BETWEEN CONCENTRATIONS (P <0.05).

The recovery times of fish exposed to 27, 70 and 500 µL L^{-1} concentrations of clove, mint, and camphor oils were 396, 329.5 and 229 s, respectively. There was no significant difference among the recorded anaesthetic recovery with regards to all of the clove oil concentrations. However, for mint and camphor oils, an increase in the concentration level produced a longer recovery time. It was also remarkable that the fish exposed to camphor had less variation in the period required to completely recover than those exposed to other products.

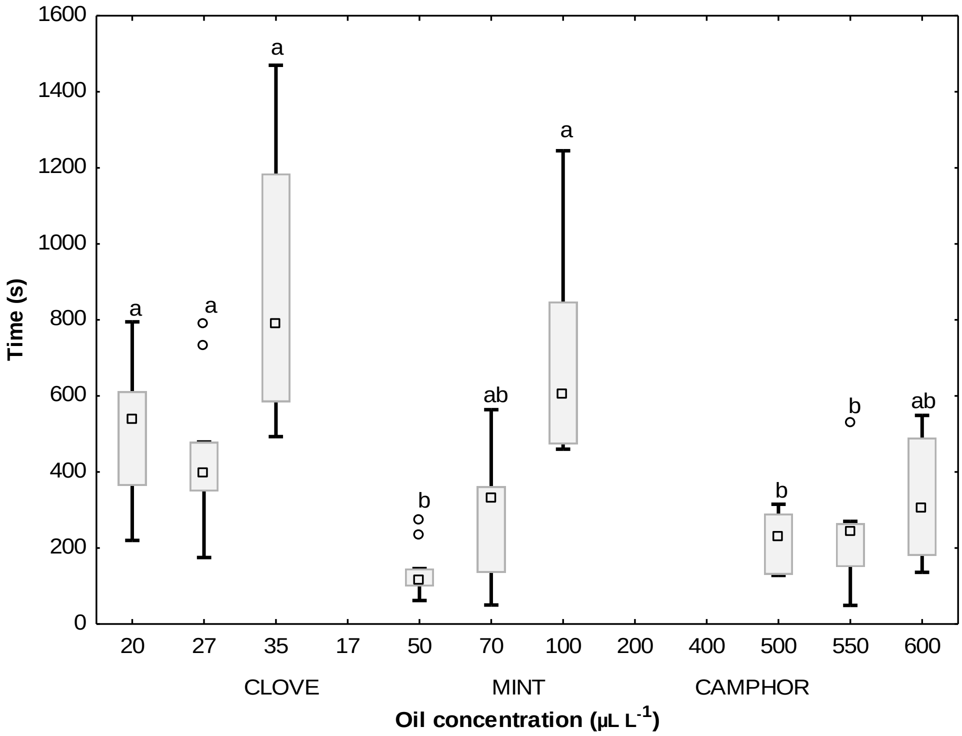

FIGURE 5. ANAESTHETIC RECOVERY (SECONDS) IN *Amphiprion ocellaris* THROUGH THE USE OF THE CLOVE, MINT AND CAMPHOR OILS. DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES BETWEEN DIFFERENT CONCENTRATIONS (P = 0.00).

Clown anemonefish subjected to 35 and 600 µL L^{-1} concentrations of clove and camphor oils re-established feeding with 24 and 48 h, respectively. There was no observed inhibition in the feeding behaviour of fish exposed to mint oil. The regression analysis indicated that the weight and length did not influence the anaesthetic induction or recovery time in any of the treatments ($p>0.05$; $r^2<0.05$).

2.4 DISCUSSION

According to Ross e Ross (2008), anaesthesia in fish should be quickly induced, and the appropriate stage should be achieved in less than three min to avoid stress and hyperactive behaviour. The recovery time should also be no longer than five min subsequent to the transfer to clean water. Considering the concentrations that provoked stage IV in most of the animals exposed without causing medullary collapse and extended recovery times, the highest concentrations were 27, 70 and 500 to 550 µL L^{-1} of clove, mint, and camphor oils, respectively. These doses did not cause mortality. The ideal clove oil concentration established in this study (equivalent to 28 mg L^{-1}) is relatively close to that (50 mg L^{-1}) found for kinguio (*Carassius auratus)* (Bittencourt et al., 2012) and pacu (*Piaractus mesopotamicus)* (Gonçalves et al., 2008*)* and is also similar (37.5 mg L^{-1}) to fat snook (*Centropomus parallelus)* (Souza et al., 2012). This is also similar to the results found by Cunha and Rosa (2006), who defined 20 mg L^{-1} as the ideal concentration for ornamental marine fishes. The same authors did not observe mortality or other adverse behavioural effects in fish after clove oil exposure. In contrast, in this study, some fish died after being exposed to 35 µL L^{-1} (equivalent to 36.43 mg L^{-1}) concentrations of clove oil, which indicates an overdose for clown anemonefish (*A. ocellaris)*. Additionally, decreased food intake was observed in the remaining fish, indicating stress promoted by the high anaesthetic concentration.

The concentration necessary for anaesthetic induction by mint oil also shows variation between species. Gonçalves *et al.* (2008) showed that 100 mg L^{-1} of menthol is the ideal concentration for pacu (*P. mesopotamicus)*. However, for tambaqui (*Colossoma macropomum)* and fat snook (*C. paralellus),* a menthol concentration of 150 mg L^{-1} was suggested by Façanha & Gomes (2005) and Souza *et al.* (2012), respectively. Moreover, for tilapia (*Oreochromis niloticus),* the concentrations that promoted surgical anaesthesia were 120 and 60 mg L^{-1} for juvenile and fingerling, respectively (Teixeira *et al.*, 2011). This correlates with the 63 mg L^{-1} concentration found to be appropriate for *A. ocellaris* in this study*.* Interestingly, the recommended mint oil concentration was almost seven times lower than the minimum needed to promote surgical anaesthesia using camphor oil (440 mg L^{-1}). Due to the large volume of camphor oil needed, volatile or water soluble substances present in this oil (easily perceived by their aroma) it may have caused an agitation that was registered in the first moments of anaesthetic induction.

Despite the similarity in the optimal concentrations of clove and mint oils necessary for anaesthesia in *A. ocellaris*, it was noted that the times were higher for both anaesthetic induction and recovery than for other species. For example, under optimal concentrations, the induction times for fat snook (*C. paralellus)* and pacu (*P. mesopotamicus)* were 92 and 134 s using clove oil and were 120 and 102 s using mint oil, respectively. Cunha and Rosa (2006) suggested that some marine ornamental species are more sensitive to anaesthetics, and frillfin goby *Bathygobius soporator* was the most resistant to clove oil of the seven species tested, requiring 180 s for induction at concentrations of 20 mg L^{-1}. In contrast, *A. ocellaris* has shown to be even more resistant, needing 310.5 and 312 s to reach stage IV with clove and mint oils, respectively. The same tendency of longer time was observed for recovery of *A. ocellaris* and were 396, 329.5 and 229 s using clove, mint and camphor oils, respectively, while other species needed less than 300 s in most situations. This discrepancy may be explained by the differences in water parameters, such as temperature and salinity, or even differences among species (Ghanawi *et al.*, 2013).

When comparing the oils tested in this study, the rapidity in which clove and mint oils promoted surgical anaesthesia is an advantage related to the use of camphor, especially for use in fast handling cases, such as biometrics procedures. It is necessary to note that while menthol provides a faster induction time, it also increases the risks involved with keeping the fish under anaesthesia for long periods of time. Induction times decreased significantly with an increase in concentration of mint oil.

According to Keene *et al.* (1998), clove oil inhibits the respiratory rate and, consequently, the ability to remove excess anaesthetic from fish, resulting in longer recovery times. Nevertheless, in longer or invasive procedures, such as artificial spawning, a longer anaesthesia period would be not only be positive but also necessary (Prince e Powell, 2000), with clove oil being the most adequate anaesthetic. Camphor oil may be particularly useful in situations when fish must be exposed to an anaesthetic for longer periods of time but require a fast recovery.

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CHAPTER 3. ANAESTHETIC EFFECT OF PROPOFOL AND MS-222 DURING CONFINEMENT OF THE CLOWNFISH *Amphiprion ocellaris* AND THEIR INFLUENCE ON WATER QUALITY

ABSTRACT

The cost and losses associated with transporting the clownfish *Amphiprion ocellaris* are considered critical points in the production chain. The aim of this study was to evaluate the anaesthetic effectiveness of propofol and MS-222 and their influence on water quality for *A. ocellaris* under confinement conditions similar to commercial transport of the species. An initial study tested the anaesthetic performance of propofol at concentrations of 0.2, 0.3 and 0.4 mg L^{-1} and MS-222 at concentrations of 10, 15 and 20 mg L^{-1} to simulate transport periods of 6, 12 and 24 hours (n = 8 fish per time/concentration). Fish were randomly selected and transferred to 16 x 30 cm polyethylene bags at a density of 5 fish L^{-1}. The pH and concentrations of dissolved oxygen (DO), total ammonia (TA-N) and gaseous ammonia (NH3-N) of the water in the plastic containers were measured immediately before and after the transport. A second experiment evaluated the use of anaesthetics at different transport densities of *A. ocellaris* (5, 10, 15 and 20 fish L^{-1}). Concentrations of 0.30 mg L^{-1} propofol and 15 mg L^{-1} MS-222 were used (n = 5 bags/density/treatment). The monitored water quality parameters were the same as those in the previous experiment. Additionally, concentrations of dissolved CO_2 in the water were measured. Although there was no evident improvement in water quality with propofol, using MS-222 at a concentration of 15 mg L^{-1} significantly reduced the production rate of metabolic waste products during the simulated transport of *A. ocellaris* at densities between 10 and 20 fish L^{-1} for periods until 24 h.

KEYWORDS: immersion anaesthesia, ornamental, reef fish, TMS, transportation.

3.1 INTRODUCTION

Marine aquariums have become increasingly popular since the end of the 20th century and beginning of the 21st century, and this popularity has been influenced by the development of technology and greater availability of products designed for the maintenance of saltwater organisms in artificial environments (Pyle, 1992; Wabnitz *et al.*, 2003). In addition, the development of this practice in recent decades has been influenced by the increasingly breeding of species with ornamental interest under controlled environmental conditions (Olivotto *et al.*, 2011). The clownfish *Amphiprion ocellaris*, the most commercialised species of ornamental reef fish, has grown substantially because of the production of individuals in captivity (Olivier, 2001; Wabnitz *et al.*, 2003; Wittenrich, 2007).

However, the greater availability of products and services naturally increases the consumer demand for healthier animals and promotes increased competition among companies in trade, which necessitates increased efficiency and a reduction of costs involved along the entire chain of production and commercialisation.

Increasing the effectiveness of the transport of ornamental marine fish is a critical point for increasing competitiveness. These animals are sensitive to high densities, and an optimal balance of conditions must be found that is economically feasible and provides for the safe transportation of fish without the risk of injury or death (Cole *et al.*, 1999). Cost is particularly important because there are often great distances involved in transporting marine fish from the producer to the end-consumer. The transportation frequently requires the use of air freight, which can reach 50-90% of the final retail value of the animal (Unep, 2008).

The use of anaesthetics during transport of ornamental fish is an option for increasing efficiency. When sedated, the fish consume less oxygen and excrete less metabolic waste, such as ammonia and CO2, which can achieve levels that endanger the survival and health of the fish (Lim *et al.*, 2003; Ross e Ross, 2008). Therefore, using anaesthetics makes it possible to transport fish at higher densities (with a consequent reduction of the volume of water transported) without impairing the water quality and animal health.

MS-222 (tricaine methanesulfonate) is the anaesthetic most widely studied and used in fish farming (Neiffer e Stamper, 2009; Topic Popovic *et al.*, 2012; Ackerman *et al.*, 2013). However, despite the current knowledge of its anaesthetic effectiveness and physiological effects in animals, few studies have evaluated the use of MS-222 for fish transport and its effects on water quality (Pramod *et al.*, 2010; Lin *et al.*, 2012). Propofol (2,6 diisopropylphenol) is a popular product for intravenous anaesthesia in non-aquatic vertebrates and has the potential for use as an anaesthetic for fish transport, mainly because of its wide safety margin (Gressler *et al.*, 2012; Gholipourkanani e Ahadizadeh, 2013). Nevertheless, no studies evaluating the effects of propofol on fish transport water quality have been conducted. In addition, no studies were found using anaesthetics during the transport of *A. ocellaris*. Therefore, the aim of this study was to evaluate the anaesthetic effectiveness of propofol and MS-222 for *A. ocellaris* and the influence of these drugs on water quality under confinement conditions similar to commercial transport of this species.

3.2 MATERIALS AND METHODS

The experiments were performed at the Laboratory for Research on Aquatic Organisms (Laboratório de Pesquisas com Organismos Aquáticos – LAPOA) of the Integrated Group for Aquaculture and Environmental Studies (Grupo Integrado de Aquicultura e Estudos Ambientais – GIA) at the Federal University of Paraná (Universidade Federal do Paraná – UFPR), which is located in Curitiba, Paraná State (PR), Brazil.

3.2.1. ANIMAL MAINTENANCE

Two hundred *A. ocellaris* juveniles were acquired from the Azul Fish Farm (São Paulo, Brazil), and they measured 0.36 ± 0.14 cm in length and 2.49 ± 0.23 g in weight (mean/standard deviation). In the laboratory, the animals were submitted to gradual acclimation of temperature, pH and salinity. After approximately 30 minutes of adaptation, they were transferred to glass tanks that measured 100 x 40 x 50 cm (length x width x height) and had black plastic film attached to the back of the tanks to reduce interference from external light. The tanks were connected to a saltwater recirculation with filtration system, and the fish were maintained in these tanks for an adaptation period of 10 days. The salinity, temperature and pH were maintained at 30 g L^{-1}, 24 ± 0.5 °C and 7.9 ± 0.02 (mean/standard deviation), respectively. Partial water changes that equalled 25% of the tank volume were performed weekly. The concentration of nitrogen as total ammonium (TA-N = N-(NH_3+NH_4^+)) were measured every three days and was maintained below 0.25 mg L^{-1}. Fish were fed *ad libitum* with a commercial pellet diet twice a day, and the pellets contained 47.5% crude protein (Tetra, Melle, Germany). An hour after feeding, any food debris and faecal material present in bottom of the tanks were removed by siphoning. Before experiments, animals were submitted to eight hours of fasting.

3.2.2. ANAESTHETIC EFFECT OF PROPOFOL AND MS-222 AND THE INFLUENCE ON WATER QUALITY

An initial factorial experiment (3 x 3) evaluated the anaesthetic effects of propofol and MS-222 on fish and their influence on *A. ocellaris* transport water. Propofol was evaluated at concentrations of 0.2, 0.3 and 0.4 mg L^{-1}; and MS-222 at concentrations of 10, 15 and 20 mg L^{-1}. These concentrations were determined based on a prior study (Pedrazzani and Ostrensky, 2014) and a pilot experiment. Stock solutions were initially prepared to obtain the respective concentrations of each anaesthetic. Propofol was diluted to 1:10 in distilled water. To dilute MS-222, 5 g of the substance was added to 1 L of saltwater (salinity of 30 g L^{-1}). All results were compared with those obtained from a control group in which fish were subjected to the same procedure but without any compound added to water.

To simulate confinement condition similar to transport, the animals (n = 8 per time/concentration) were randomly selected from the maintenance aquariums and transferred to 16 x 30 cm polyethylene bags at a density of 5 fish L^{-1}. The bags were then filled with 400 mL of saltwater and pure oxygen in a 1:2 ratio, and anaesthetic was added at the respective experimental concentrations. These bags were sealed with rubber bands and placed into isothermal boxes where were kept for 6, 12 and 24h.

The physicochemical parameters indicative of water quality inside the plastic bags were measured immediately before the injection of oxygen and sealing of the bags (initial values) and immediately after opening the bags, later than transport period (final values). The pH was measured using a digital benchtop pH meter (AZ 86505, Taiwan). The concentration of dissolved oxygen and the water temperature were measured using a digital oximeter (YSI 550A, USA). The initial and final concentrations of TA-N were determined by the indophenol method (Apha, 2005a), which was followed by determination by spectrophotometry (Spectronic Genesys 20, England). The concentration of gaseous ammonia (NH_3-N) was calculated using the formula proposed by Ostrensky *et al.* (1992), based on the values for pH, temperature, salinity and TA-N concentration. The initial DO concentration, temperature and pH of the water before the experiment were 6.20 ± 0.27 mg.L^{-1}, 23.0 ± 0.38 °C and 7.95 ± 0.03 (mean ± standard deviation), respectively, and the initial concentrations of TA-N were below the detection limit of the method used.

After opening the plastic bags, the individual anaesthetic stage was assessed, being classified as anaesthetic stages I: Absence of reaction to touch and to visual stimulus; II: Initial loss of balance, characterized by difficulty to maintain normal swimming position; III: Total loss of balance, uncoordinated swimming; IV: Minimal opercular movement and no swimming; V: no opercular beating, medullar collapse, eventual death, according to Ross and Ross (2008). The sedation (stage II) was considered the desirable anaesthetic stage for transport proposes. The animals with behavioural signs of anaesthesia were monitored for the period necessary for anaesthesia recovery. Fish with normal position and swimming were considered recovered. During the subsequent 72 hours, animals were transferred to a 900 L saltwater recirculation system, divided into 15 plastic tanks with a 60 L capacity, where the presence of injuries and fish mortality were recorded. This system featured environmental conditions similar to those found in the maintenance tanks.

3.2.3. EFFECT OF FISH DENSITY ON WATER QUALITY USING ANAESTHETICS

A second experiment evaluated the influence of fish density on water quality during confinement condition, similar to transport of *A. ocellaris,* using propofol and MS-222. Four densities were evaluated for each anaesthetic. These densities were determined from a previous experiment and commercial practices routinely adopted for transporting clownfish in Brazil. To obtain that, five fish were placed in each bag (4 replicates/ treatment) and the volume of water containing anaesthetic was reduced according to the density increased. For the densities simulation of 5, 10, 15 and 20 fish L^{-1}, were added 1.000 mL, 500 mL, 333mL and 250 mL, respectively, of clean saltwater. The concentrations of propofol and MS-222 were 0.30 and 15 mg L^{-1}, respectively. Results were compared with those obtained in a control group in which no substance was added to the water.

The methodology used to simulate confinement conditions of transport and to measure the physicochemical parameters of water was similar to that described in Section 3.2.2. Additionally, the concentration of dissolved carbon dioxide (CO_2) in water was measured by colorimetry using sodium hydroxide and phenolphthalein (Apha, 2005b) before and after the 24h period. The initial DO and CO_2 concentrations, temperature and pH of the water were 6.30 ± 0.18 mg L^{-1}, 5.80 ± 0.37 mg L^{-1}, 24.3 ± 0.53 °C and 7.9 ± 0.06 (mean ± standard deviation), respectively. The initial concentrations of TA-N and, consequently, of NH_3-N remained below the detection limit of the method used. The mortality and ingestion of food were also evaluated during the 72 hours following the experiment.

3.2.4. STATISTICAL ANALYSIS

The normality of data was assessed by the Shapiro-Wilk test. Because data did not fit a normal (Gaussian) curve, the statistical differences between analysed variables were assessed by the Kruskal-Wallis test ($p<0.05$). Results for the anaesthetic at different concentrations and transport times were analysed separately.

Subsequently, the anaesthetic performances of different treatments were compared among each other in terms of transport water quality parameters at diverse densities using a linear regression analysis. Analyses were performed using the Statistica version 10.0 software (Statsoft, Tulsa, USA).

3.3. RESULTS

3.3.1. ANAESTHETIC EFFECT AFTER DIFFERENT CONFINEMENT PERIODS

The results showed that MS-222 induced anaesthesia to stage II only when used at the two highest concentrations and/or longest exposure times (TABLE 7). In propofol treatments, fish at all of the concentrations were induced to Stage II anaesthesia. Only fish treated with 0.4 mg L^{-1} for 6 hours showed signs of stage III anaesthesia.

TABLE 7. ANAESTHETIC STAGES REACHED AND MORTALITY (N) AFTER TRANSPORT PERIODS FOR *Amphiprion ocellaris* USING MS-222 AND PROPOFOL AT THEIR RESPECTIVE CONCENTRATIONS AND THE CONTROL TREATMENT.

Treatment	Concentration (mg L^{-1})	Period		
		06h	12h	24h
Control	0	–	–	–
MS-222	10	–	–	II
	15	–	II	II
	20	–	II (2)	II
Propofol	0.2	II	II	II
	0.3	II	II	II
	0.4	III (1)	II	II

Only two deaths were observed for the treatment with 20 mg L^{-1} of MS-222 (12 h), and there was one death when using propofol at a concentration of 0.40 mg L^{-1} (6 h). Although animals subjected to propofol at all concentrations recovered within 10 min after being transferred to water without the anaesthetic, after the transport periods, fish subjected to MS-222 at the highest concentration kept swimming erratically and showed partial loss of equilibrium for up to eight hours.

3.3.2. INFLUENCE OF ANAESTHETICS ON WATER QUALITY

In all treatments, there was a higher final DO concentration in because pure oxygen was injected immediately prior to sealing plastic bags (TABLE 8). Therefore, this final concentration was always above 9.5 mg L^{-1}. There was a tendency to reduce pH values over the same period. In the control treatment, water acidification occurred mainly in the period between 12 and 24 h of transport, during which the reduction in pH was 0.26. In treatments containing propofol and MS-222, the most significant decreases in pH values occurred between 6 and 12 h, with decreases from 0.78 to 0.67 detected in the treatments of 0.3 and 10 mg L^{-1} of each substance, respectively.

In the control treatment, there was an increase in TA-N and NH_3-N concentrations that was directly proportional to the transport period (TABLE 9). In propofol treatments, there was also an increase in these concentrations (6 h and 24 h); however, the near zero values obtained after 12 h were significantly lower than those from the other periods analysed. A similar trend was observed in the 20 mg L^{-1} of MS-222 treatment, which showed higher concentrations of TA-N and NH_3-N in the first 12 h, which decreased in 24 h of transport.

TABLE 8. VALUES (MEDIAN, MINIMUM AND MAXIMUM) FOR DISSOLVED OXYGEN (DO) AND pH IN WATER AFTER THE VARIOUS TRANSPORT PERIODS FOR *Amphiprion ocellaris* USING PROPOFOL, MS-222 AND THE CONTROL TREATMENT.

Treatment	Concentration ($mg\ L^{-1}$)	DO ($mg\ L^{-1}$) 06h	DO 12h	DO 24h	pH 6h	pH 12h	pH 24h
Control	0	17.75 ^{a} (12.3 – 18.9)	18.85 ^{b} (18.4 – 20.0)	19.20 ^{b} (18.3 – 20.0)	7.83 ^{a} (7.45 – 7.87)	7.60 ^{a} (7.50 – 7.60)	7.34 ^{b} (7.31 – 7.45)
Propofol	0.2	12.55 $^{a\ A}$ (10.4 – 16.9)	13.25 ^{a} (12.2 – 15.7)	16.85 $^{b\ A}$ (13.7 – 17.3)	7.54 ^{A} (7.22 – 7.8)	7.26 ^{A} (7.34 – 7.28)	7.28 ^{A} (7.15 – 7.30)
	0.3	10.10 $^{a\ B}$ (9.6 – 10.4)	13.70 ^{ab} (9.6 – 15.3)	13.85 $^{b\ B}$ (12.1 – 16.3)	7.96 $^{a\ AB}$ (7.89 – 8.00)	7.17 $^{b\ B}$ (7.11 – 7.21)	7.29 $^{ab\ AB}$ (7.28 – 7.32)
	0.4	10.70 $^{a\ AB}$ (9.6 – 12.7)	12.55 ^{a} (10.2 – 14.0)	15.10 $^{b\ AB}$ (14.2 – 15.7)	8.03 $^{a\ B}$ (8.02 – 8.04)	7.21 $^{b\ A}$ (7.19 – 7.23)	7.31 $^{ab\ B}$ (7.28 – 7.32)
MS-222	10	17.65 ^{A} (16.7 – 22.5)	18.60 (15.7 – 19.2)	16.65 (15.8 – 19.8)	7.39 ^{A} (7.06 – 7.45)	7.31 ^{A} (7.00 – 7.41)	7.37 ^{A} (7.32 – 7.47)
	15	18.80 ^{A} (15.0 – 19.5)	18.65 (18.3 – 20.5)	17.40 (15.5 – 20.8)	7.55 $^{a\ AB}$ (7.52 – 7.60)	7.51 $^{b\ B}$ (7.50 – 7.53)	7.51 $^{b\ B}$ (7.49 – 7.52)
	20	15.45 $^{a\ B}$ (14.3– 15.9)	16.30 ^{ab} (13.3 – 18.8)	17.35 ^{b} (16.4 – 18.8)	7.63 $^{a\ B}$ (7.62 – 7.65)	7.47 $^{b\ B}$ (7.46 – 7.50)	7.37 $^{b\ A}$ (7.32 – 7.47)

NOTE: DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES ($P<0.05$) BETWEEN TRANSPORT TIMES (HORIZONTAL; LOWERCASE) AND BETWEEN VARIABLES FOR THE DIFFERENT CONCENTRATIONS IN THE SAME TRANSPORT PERIOD (VERTICAL; UPPERCASE).

TABLE 9. VALUES (MEDIAN, MINIMUM AND MAXIMUM) FOR CONCENTRATIONS OF TOTAL AMMONIA (TA-N) AND GASEOUS AMMONIA (NH_3-N) IN WATER AFTER DIFFERENT TRANSPORT PERIODS FOR *Amphiprion ocellaris* USING MS-222, PROPOFOL AND NO ANAESTHETIC (CONTROL TREATMENT).

Treatment	Concentration	TA-N (mg L^{-1})			NH_3-N (mg L^{-1})		
	(mg L^{-1})	06h	12h	24h	06h	12h	24h
Control	0	0.73 ^{ab}	0.50 ^{a}	1.20 ^{b}	0.12 ^{ab}	0.07 ^{a}	0.13 ^{b}
		(0.25 – 1.28)	(0.49 - 0.50)	(0.30 – 1.90)	(0.04 – 0.16)	(0.07)	(0.06 – 0.23)
Propofol	0.2	0.09 ^{a}	0.00 ^{b}	0.01 $^{ab\ A}$	0.01 $^{a\ A}$	0.00 ^{b}	0.00 $^{ab\ A}$
		(0 – 0.29)	(0.00)	(0.00 – 0.07)	(0 – 0.03)	(0.00)	(0 – 0.01)
	0.3	0.07 ^{ab}	0.00 ^{a}	0.40 $^{b\ AB}$	0.01 $^{ab\ A}$	0.00 ^{a}	0.04 $^{b\ AB}$
		(0 – 0.20)	(0.00)	(0.00 – 0.56)	(0 – 0.04)	(0.00)	(0 – 0.05)
	0.4	0.18 ^{ab}	0.00 ^{a}	0.58 $^{b\ B}$	0.03 $^{a\ B}$	0.00 ^{b}	0.05 $^{a\ B}$
		(0.13 – 0.24)	(0.00)	(0.32 – 0.88)	(0.02 – 0.05)	(0.00)	(0 - 0.08)
MS-222	10	0.38 ^{A}	0.36	0.56 ^{AB}	0.04 ^{AB}	0.03	0.05 ^{AB}
		(0.23 – 0.64)	(0.08 – 0.78)	(0.34 – 0.76)	(0.02 – 0.07)	(0.01 – 0.07)	(0.03 – 0.08)
	15	0.37 $^{a\ A}$	0.37 ^{a}	0.94 $^{b\ A}$	0.05 $^{a\ A}$	0.04 ^{a}	0.11 $^{b\ A}$
		(0.28 – 0.60)	(0.14 – 0.49)	(0.65 – 1.76)	(0.03 – 0.07)	(0.02 – 0.06)	(0.07 – 0.19)
	20	0.10 ^{B}	0.42	0.08 ^{B}	0.01 ^{B}	0.05	0.01 ^{B}
		(0 – 0.40)	(0.31 – 0.47)	(0 – 0.49)	(0 – 0.05)	(0.03 – 0.05)	(0 - 0.05)

NOTE: DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES ($p<0.05$) BETWEEN TRANSPORT TIMES (HORIZONTAL; LOWERCASE) AND BETWEEN DIFFERENT CONCENTRATIONS FOR THE SAME ANAESTHETIC (VERTICAL; UPPERCASE).

3.3.3. POOLED ANALYSIS OF THE EFFECTS OF ANAESTHETICS ON WATER QUALITY OVER TIME

Analysis of the data obtained at all of the concentrations of each substance in a pooled and sequential manner (FIGURE 6) revealed that the DO concentrations in transport water exhibited smaller increases in propofol treatment. Moreover, treatments containing this anaesthetic showed a marked decline in pH 12 h after starting the experiment. Water acidification was more gradual in the other treatments. The concentrations of TA-N and NH_3-N for the control treatment increased in the first six hours and then declined. In the treatment with propofol, the concentrations of TA-N and NH_3-N remained significantly below those recorded in the control and MS-222 treatments. In MS-222, the TA-N and NH_3-N concentrations increased gradually over 24 h.

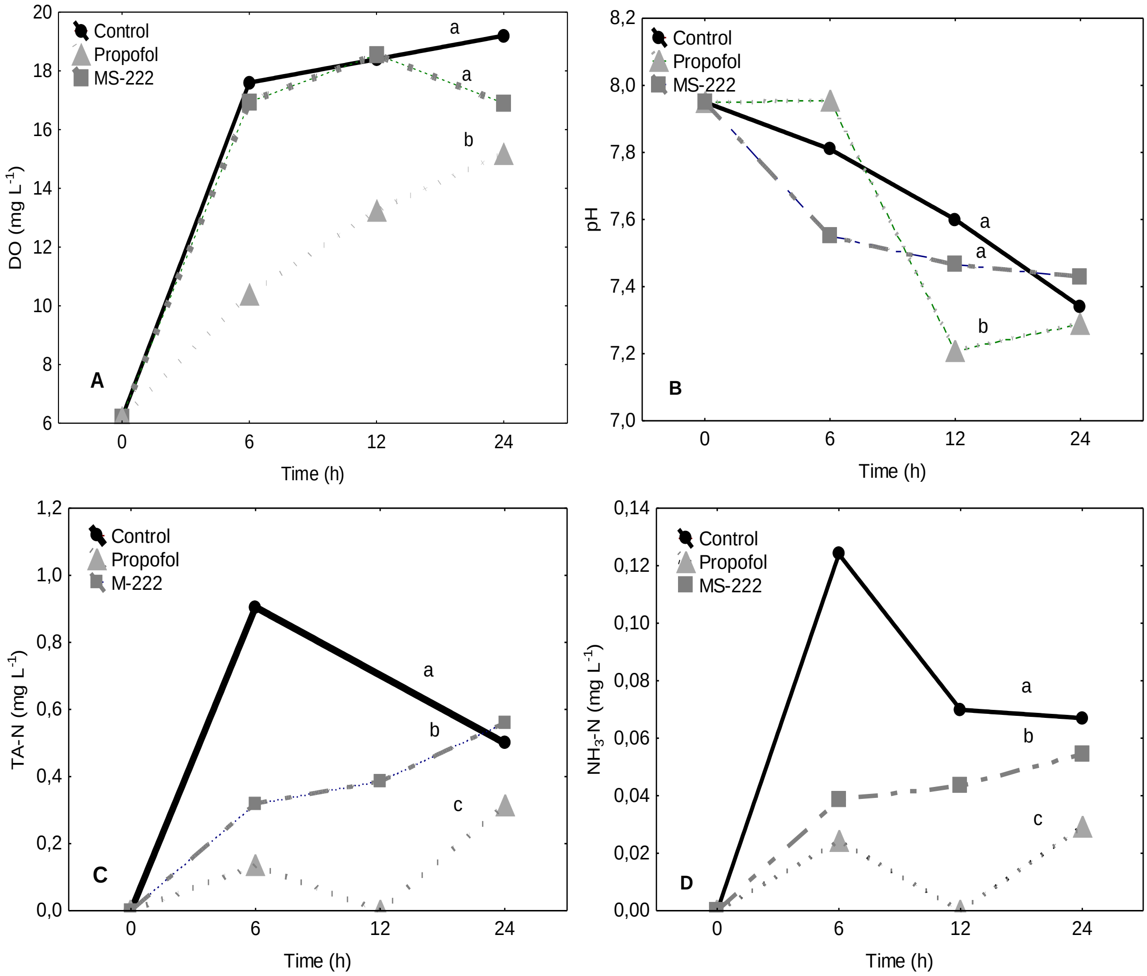

FIGURE 6. CONCENTRATIONS (MEDIANS) OF DISSOLVED OXYGEN (A), pH (B), TOTAL AMMONIA (C) AND GASEOUS AMMONIA (D) IN THE TRANSPORT WATER OF *Amphiprion ocellaris* USING MS-222, PROPOFOL AND THE CONTROL (NO ANAESTHETIC).

3.3.4. EFFECTS OF FISH DENSITY ON WATER QUALITY USING DIFFERENT ANAESTHETICS

In all treatments, there was a tendency of increase final concentrations of DO and CO_2 when using higher densities in simulated transport (TABLE 10).The concentrations of TA-N and NH_3-N in control and propofol treatments were also significantly higher for densities starting from 10 fish L^{-1}, reaching 4.29 and 0.36 mg L^{-1} and 4.17 and 0.34 mg L^{-1} (medians) in the treatments containing 20 fish L^{-1} for the control and propofol treatments, respectively. In the MS-222 treatment, there was a reduced trend toward higher ammonia concentrations, with stabilisation of TA-N and

NH_3-N concentrations occurring at 0.54 and 0.05 mg L^{-1}, respectively, in the treatment containing 20 fish L^{-1}. In this same density, acidification of the water was observed in all treatments, with the greatest reduction in pH (0.89) recorded in the control and the smallest decrease (0.37) in MS-222 treatment.

TABLE 10. CONCENTRATIONS (MEDIAN, MINIMUM AND MAXIMUM) OF DISSOLVED OXYGEN (DO), DISSOLVED CARBON DIOXIDE (CO_2), TOTAL AMMONIA (TA-N), GASEOUS AMMONIA (NH_3-N) AND pH IN WATER 24 HOURS AFTER TRANSPORT WITH DIFFERENT *Amphiprion ocellaris* DENSITIES USING PROPOFOL, MS-222 AND THE CONTROL TREATMENT.

Treatment	**Density (Fish L^{-1})**	**DO (mg L^{-1})**	**CO_2 (mg L^{-1})**	**TA-N (mg L^{-1})**	**NH_3- N (mg L^{-1})**	**pH**
Control	05	14.00 ^{a}	05.0 ^{a}	0.57 ^{a}	0.06 ^{a}	7.24 ^{a}
		(11,8 – 15.0)	(5.0 – 8.0)	(0.35 – 1.01)	(0.03 – 0.10)	(7.24 – 7.33)
	10	16.90 ^{b}	09.5 ^{b}	2.59 ^{b}	0.19 ^{b}	6.97 ^{b}
		(15.4- 17.8)	(7.0 – 12.0)	(2.15 – 2.73)	(0.16 – 0.20)	(6.93 – 6.98)
	15	17.10 ^{b}	11.0 ^{bc}	4.05 ^{c}	0.31 ^{c}	7.01 ^{c}
		(16.0 – 17.6)	(10.0 – 12.0)	(3.74 – 4.71)	(0.30 – 0.36)	(7.00 – 7.05)
	20	17.70 ^{b}	13.5 ^{c}	4.29 ^{c}	0.36 ^{c}	7.09 ^{c}
		(16.3 – 19.2)	(11.0 – 18.0)	(3.79 – 5.29)	(0.29 – 0.39)	(6.99 – 7.15)
MS-222 (mg L^{-1})	05	17.50 ^{a}	06.0 ^{a}	0.50 ^{a}	0.05 ^{a}	7.53
		(15.3 – 19.4)	(5.0 – 6.0)	(0.17 – 0.66)	(0.02 – 0.07)	(7.52 – 7.54)
	10	17.80 ^{a}	07.0 ^{b}	0.70 ^{b}	0.09 ^{b}	7.10 ^{a}
		(15.9 – 19.4)	(6.0 – 9.0)	(0.52 – 1.30)	(0 – 0.11)	(7.07 – 7.10)
	15	19.50 ^{b}	07.0 ^{b}	1.15 ^{b}	0.10 ^{b}	7.13 ^{a}
		(18.0 – 20.6)	(6.0 – 9.0)	(0.88 – 1.41)	(0.07 – 0.11)	(7.12 – 7.13)
	20	18.00 ^{ab}	7.5 ^{b}	0.54 ^{ab}	0.05 ^{ab}	7.12 ^{b}
		(16.7 – 19.5)	(7.0 – 9.0)	(0.37 – 0.72)	(0.03 – 0.06)	(7.10 – 7.12)
Propofol (mg L^{-1})	05	16.00 ^{a}	06.0 ^{a}	0.00 ^{a}	0.00 ^{a}	7.31 ^{a}
		(12.6 – 16.8)	(5.0 – 7.0)	(0 – 0)	(0 – 0)	(7.28 – 7.32)
	10	15.0 ^{a}	07.5 ^{b}	1.85 ^{b}	0.15 ^{b}	7.04 ^{b}
		(12.1 - 17.1)	(6.0 - 9.0)	(1.75 – 2.41)	(0.14 – 0.20)	(7.01 – 7.05)
	15	16.35 ^{a}	08.5 ^{bc}	3.24 ^{c}	0.27 ^{c}	7.05 ^{c}
		(14.8 – 17.5)	(7.0 – 10.0)	(2.25 – 3.92)	(0.19 – 0.33)	(7.05 – 7.06)
	20	17.80 ^{b}	7.0 ^{ab}	4.17 ^{c}	0.34 ^{c}	7.03 ^{b}
		(16.0 – 18.9)	(5.0 – 9.0)	(2.08 – 4.58)	(0.17 – 0.37)	(7.01 – 7.05)

NOTE: DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES BETWEEN DIFFERENT DENSITIES IN THE SAME TREATMENT (VERTICAL) ($P<0.05$).

3.3.5. POOLED ANALYSIS OF THE ANAESTHETICS EFFECTS ON THE WATER QUALITY AT DIFFERENT TRANSPORT DENSITIES

Pooled analysis of the data obtained at different densities and for each treatment revealed that the control and propofol treatments exhibited the highest DO concentrations (FIGURE 7). However, the control treatment also featured higher CO_2 concentrations, with a strong positive correlation between CO_2 and the tested density (TABLE 11). Although all of the treatments showed significant acidification of the water starting at a density of 10 fish L^{-1}, the MS-222 had a smaller pH change compared to the other treatments. The control and propofol exhibited a greater effect of density on the TA-N and NH_3-N concentrations in the transport water. In contrast, in the MS-222 treatment, TA-N and NH_3-N concentrations were relatively stable, resulting in a lack of correlation between the measured values and tested densities.

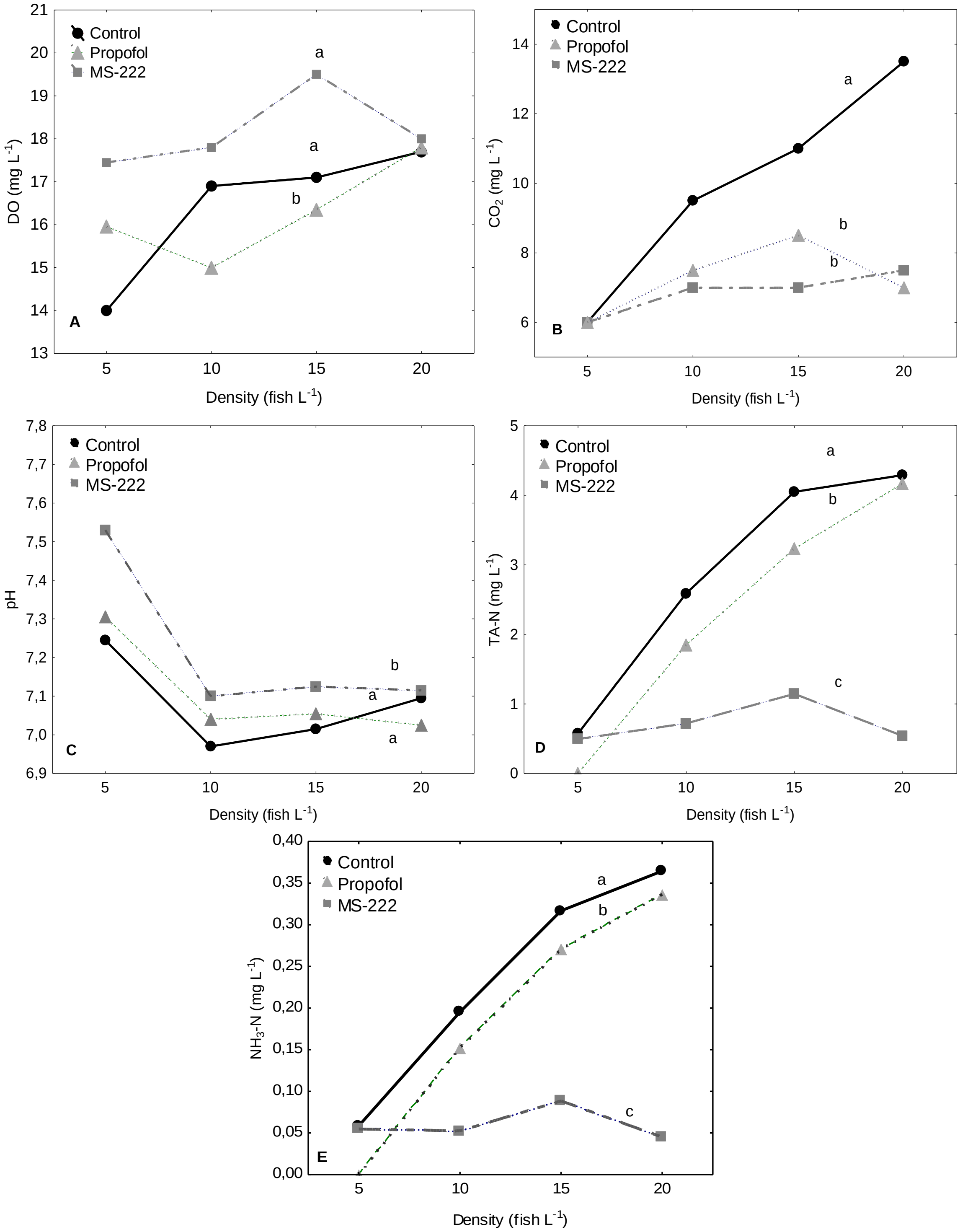

FIGURE 7. (A) VARIATIONS IN DISSOLVED OXYGEN (DO), (B) CARBON DIOXIDE (CO_2), (C) pH, (D) TOTAL AMMONIA (TA-N) AND (E) GASEOUS AMMONIA (NH_3-N) IN WATER, USING MS-222, PROPOFOL AND NO ANAESTHETIC (CONTROL) DURING 24 H TRANSPORT PERIOD. DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES BETWEEN TREATMENTS ($p<0.05$).

TABLE 11. RESULTS OF THE LINEAR CORRELATION ANALYSIS BETWEEN DENSITIES USED FOR EACH TREATMENT AND DETERMINANT VARIABLES OF WATER QUALITY.

Variable	Treatment	Tendency	r^2	p	Correlation
	Control	+	0.77	0.11	NS
DO	Propofol	+	0.58	0.23	NS
	MS-222	+	0.22	0.52	NS
	Control	+	0.97	0.01	Very strong*
CO_2	Propofol	+	0.24	0.50	NS
	MS-222	+	0.85	0.07	NS
	Control	-	0.18	0.56	NS
pH	Propofol	-	0.57	0.24	NS
	MS-222	-	0.64	0.19	NS
	Control	+	0.90	0.04	Very strong*
TA-N	Propofol	+	0.97	0.01	Very strong*
	MS-222	+	0.05	0.75	NS
	Control	+	0.96	0.01	Very strong*
NH_3-N	Propofol	+	0.97	0.01	Very strong*
	MS-222	+	0.00	0.95	NS

NOTE: CORRELATIONS WITH r^2 GREATER THAN 0.90 WERE CONSIDERED VERY STRONG. NS: NOT SIGNIFICANT.

3.4. DISCUSSION

To achieve the desired anaesthetic stage, a combination of factors should be considered, including anaesthetic agent employed, concentration to be used and duration of exposure to the anaesthetic (Ibarra-Zatarain *et al.*, 2011). The optimal anaesthetic stage to be reached by the fish varies according to the procedure to which the animals are subjected, such as biometrics, transport or surgery (Ross e Ross, 2008). Stage II anaesthesia is adequate for maintaining animals during transport because sedation occurs at this stage without a total loss of equilibrium, thereby avoiding the overlapping of animals at the bottom of the container, which may cause injuries and suffocation (Coyle *et al.*, 2004).

Therefore, it is expected that *A. ocellaris* reach deeper anaesthetic stages with increasing anaesthetic concentration and duration of exposure, and this expectation was verified for the use of MS-222. However, fish subjected to propofol suffered the opposite effect, as the exposure time increased, the anaesthetic level decreased. The increasing degree of anaesthesia over time caused by exposure to MS-222 and its longer period of recovery from anaesthesia can be explained by the fact that the product can accumulate in various organs and tissues, remaining in the body for up to 24 h after exposure (Ross e Ross, 2008). Propofol, however, is known as a short-acting anaesthetic that is rapidly metabolised by the body and characterised by causing a lack of cumulative effects and rapid recovery (Fish *et al.*, 1997; Fleming *et al.*, 2003; Gholipourkanani e Ahadizadeh, 2013). Considering these characteristics of metabolism of the respective anaesthetics in addition to an association between exposure time, concentration and the desired anaesthetic stage, the optimal concentrations for sedation of *A. ocellaris* during transport for periods until 24 h would be 15 and 0.3 mg L^{-1} of MS-222 and propofol, respectively.

This study showed that under transport confinement conditions, even for short periods, the fish metabolism directly affects the water quality, and deleterious effects of this interaction can be minimised by using anaesthetics. The DO concentrations in water exhibited an increasing tendency over time in all treatments. This result clearly shows that the volume of oxygen injected into the transport bags exceeds the uptake capacity of animals over short time periods, because the diffusion of the substance to water. Similar findings have been reported in other studies involving the use of anaesthetics during transport of the guppy *Poecilia reticulata* (Teo e Chen, 1993), platy *Xiphophorus maculatus* (Guo *et al.*, 1995) and angel fish *Pterophyllum scalare* (Chellapan *et al.*, 2013). In the present study, however, there was an increase in the final DO concentrations in the treatments with the highest fish density. The increased CO_2 concentrations observed in the higher density treatments occurred as expected because when animals are confined in the same environment, metabolic waste production is directly proportional to the number of individuals (Cole *et al.*, 1999). According to Pramod *et al.* (2010), increasing CO_2 concentrations may reduce the capacity for oxygen transport by haemoglobin in the blood of fish, which would explain the higher final DO concentration in the treatments with higher fish densities.

The decreased pH observed in all treatments resulted in lower proportions of NH_3 relative to NH_4^+ because the rate of ionisation decreases with decreasing pH, resulting in a lower proportion of ammoniacal nitrogen in its most toxic form in water (Ostrensky e Wasielesky Jr, 1995; Lim *et al.*, 2003). However, with the increasing concentrations recorded for MS-222, the pH tended toward neutrality. Although MS-222 is a known acidifier in freshwater that has a low alkalinity, when MS-222 is used in saltwater, its buffering effect is usually sufficient to reduce the pH variation (Carter *et al.*, 2011; Topic Popovic *et al.*, 2012). The largest drop in pH was observed in the treatment with 0.3 mg L^{-1} of propofol and coincided with the period in which the anaesthetic level decreased (transition from stage III to II). This water acidification may have hindered the absorption of the anaesthetic because it is known that in immersion anaesthetic solutions, a reduction in pH increases the ionisation of the substance and reduces its effectiveness (Neiffer e Stamper, 2009).

In the first experiment at a density of 5 fish L^{-1}, the concentrations of TA-N and NH_3-N in the propofol treatment generally remained numerically below the values observed in the MS-222 and control treatments. However, this situation was reversed when the fish density increased. In the second experiment, the treatments subjected to MS-222 exhibited lower concentrations of TA-N and NH_3-N than did the propofol and control treatments despite the water remaining more alkaline than in the other treatments. All these results indicate a reduction in fish metabolism threatened with MS-222.

The strongly positive correlations between the control treatment and concentrations of CO_2, TA-N and NH_3-N suggest intense metabolic activity of fish during transport. Propofol also featured strong correlations with the concentrations of TA-N and NH_3-N, which indicated that there was also increased metabolic activity that most likely occurred during the anaesthetic recovery phase, although the increase was at low levels and not reflected in a significant increase in CO_2 production such as occurred in the control. MS-222 was more effective in reducing metabolic activity, thereby leading to a decline in the elimination rate of these toxic wastes inside the bags and delaying the deleterious effects of time on changes in transport water quality.

Propofol in a concentration of 0.3 mg L^{-1} presented significant effects of N-AT and N-NH_3 reduction at low stocking densities (5 fish L^{-1}). However, MS222 at a concentration of 15 mg L^{-1} showed better results than propofol under the same fish confinement conditions at higher densities (10 to 20 fish L^{-1}). MS-222 effectively reduced the water metabolic wastes and maintained the monitored *A. ocellaris.* water quality parameters during the confinement period of 24 h.

3.5 ACKNOWLEDGMENTS

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CHAPTER 4. EFFECT OF USING ESSENTIAL OILS OF CLOVE, MINT AND CAMPHOR ON CONFINED CLOWN FISH *Amphiprion ocellaris* (CUVIER 1830) AND THEIR INFLUENCE ON WATER QUALITY

ABSTRACT

The conditions under which marine ornamental fish are transported directly influence the health, well-being and viability of the animals. The aim of the present study was to evaluate the anaesthetic efficacy of the essential oils of clove, mint and camphor on clownfish *Amphiprion ocellaris* and their effects on water quality in simulated transport conditions. In a first experiment, the anaesthetic effects of clove, mint and camphor oils were tested at concentrations of 2.5, 5.0 and 7.5 µL L^{-1}; 20, 25 and 30 µL L^{-1}; and 100, 120 and 140 µL L^{-1}, respectively. Travelling times of 6, 12 and 24 hours were simulated (n=8 fish per time/concentration). Animals were randomly selected and transferred to polyethylene bags measuring 16 x 30 cm at a density of 5 fish L^{-1}. The pH and concentrations of dissolved oxygen (DO), total ammonia (N-TA) and gaseous ammonia (N-NH_3) of water in the plastic bags were measured immediately before closing and after opening the bags. A second experiment evaluated the use of essential oils at different confinement densities of *A. ocellaris* (5, 10, 15 and 20 fish L^{-1}). For that, bags containing 5 fish and 1.000 mL, 500 mL, 333mL and 250 mL of water and the anaesthetics were placed in isothermal boxes (4 replicates/ treatment). Concentrations of 5, 25 and 120 µL L^{-1} were used for clove, mint and camphor oils, respectively for 24 h of confinement. The water-quality parameters monitored were the same as in the previous experiment, with the addition of measuring concentrations of dissolved CO_2 in the water. Using mint oil at a concentration of 25 µL L^{-1} and a maximum density of 10 fish L^{-1} significantly reduced the concentration of N-TA, thus favouring the use of these oils during transport of *A. ocellaris*. At low densities (5 fish L^{-1}) clove and camphor oils at concentrations of 5 and 120 µL $L^{-1,}$ respectively, can also be used safely for confinement of 24 h.

KEYWORDS: Immersion anaesthesia, ornamental, plant oils, reef fish, transportation.

4.1 INTRODUCTION

Marine ornamental fish are captured and traded by at least 45 countries, located mainly in tropical and subtropical zones (Bartley, 2000). According to FAO data, emerging countries export 63% of the worldwide total reef fish, with one third from the Philippines, one third from Indonesia and the rest from countries such as the Maldives, Vietnam, Thailand, Sri Lanka, Puerto Rico, Australia, Hawaii and Brazil (Bruckner, 2005). The major importers are the United States, the European Union and Japan (Pomeroy *et al.*, 2006), and main ornamental species involved in this trade, in terms of number of individuals sold, is the clown anemonefish *Amphiprion ocellaris* (Green, 2008).

Because there are generally large geographic distances separating the collection and/or cultivation sites and the points of sale, and because the fish must arrive in good condition at their destination, the productive chain of ornamentals requires complex logistics for transportation and distribution. The conditions under which the fish are transported, in turn, directly influence the health and viability of traded animals and, consequently, the economic efficiency of the operation (Unep, 2008).

The greatest challenges during the transport of ornamental fish, especially when performed over long distances and at high densities, are the avoidance of excessive stress, mechanical shocks and deterioration of water quality caused by the elimination of metabolic waste from the fish, mainly ammonia and carbon dioxide (Leitritz, 1969; Cole *et al.*, 1999; Lin *et al.*, 2012). Because ornamental fish are packed in small volumes of water under transport conditions, organic wastes can accumulate and reach toxic levels (Lim *et al.*, 2003).

Sedation is one option used to minimise stress and possible injuries caused by the agitated state of fish during transport (Cooke *et al.*, 2004; Coyle *et al.*, 2004; Pramod *et al.*, 2010). Anaesthesia reduces metabolic rates and consequently the oxygen uptake and excretion of metabolic products into the water throughout the transport period (Ross e Ross, 2008). The selection of products use during transport often depends on factors such as availability, cost-effectiveness, ease of use and safety of handlers (Cho e Heath, 2000). These factors make some essential oils potentially suitable for anaesthesia of fish during confinement conditions of transport.

The clove *Eugenia caryophyllata* essential oil has been studied as a sedative to minimise the effects of transportation on marbled spinefoot *Siganus rivulatus* (Ghanawi *et al.*, 2013), largemouth bass *Micropterus salmoides* (Cooke *et al.*, 2004), Mongolian redfin *Culter mongolicus* (Lin *et al.*, 2012) and angelfish *Pterophyllum scalare* (Chellapan *et al.*, 2013). The mint *Mentha arvensis* and camphor *Cinnamomum camphora* essential oils have proven anaesthetic effects on clownfish *Amphiprion ocellaris* and can be used during animal laboratory handling (Pedrazzani e Ostrensky, 2014). The aim of the present study was to evaluate the anaesthetic efficacy of clove, mint and camphor essential oils on clown anemonefish *Amphiprion ocellaris* and their effects on water quality under confinement conditions similar to transport.

4.2 MATERIALS AND METHODS

The experiments were performed at the Laboratory for Research on Aquatic Organisms (Laboratório de Pesquisas com Organismos Aquáticos - LAPOA) of the Integrated Group of Aquaculture and Environmental Studies (Grupo Integrado de Aquicultura e Estudos Ambientais - GIA) at the Federal University of Paraná (Universidade Federal do Paraná - UFPR), located in Curitiba, Paraná State, Brazil.

4.2.1 ANIMAL CARE

Two hundred juvenile *A. ocellaris* produced by the company Azul Fish Farm (São Paulo, Brazil) were acquired. The fish, with a total length of 2.75 ± 0.39 cm (mean/standard deviation) and weight of 0.47 ± 0.42 g (mean/standard deviation), were transported at a density of 10 fish L^{-1} of water in plastic bags containing water and pure oxygen in a 1:2 ratio. The bags were packed in isothermal boxes and transported by air. The total duration of the process, from packing the animals until their arrival in the laboratory, was seven hours.

In laboratory, animals underwent gradual acclimation to the desired temperature, pH and salinity. After approximately 30 minutes of adaptation, the fish were transferred to glass tanks measuring 100 x 40 x 50 cm (length x width x height) with black plastic film attached to the back of the tank to reduce interference from external light. Tanks were connected to a saltwater recirculation system. Fish were kept in these tanks for 10 days. The salinity, temperature and pH were maintained at 30 g L^{-1}, 24 ± 0.5ºC and 7.9 ± 0.02 (mean/standard deviation), respectively. Partial water changes of 25% of the tank volume were performed weekly. The concentrations of nitrogen in the form of total ammonium [(N-TA = N-(NH_3+NH_4^+)] were measured every three days and always kept at levels below 0.25 mg L^{-1}. The fish were fed twice daily *ad libitum* with a commercial pellet diet containing 47.5% crude protein (Tetra®, Melle, Germany). One hour after feeding, leftover food and faecal matter present in tanks were removed by siphoning.

4.2.2 ANAESTHETIC EFFECT AND INFLUENCE OF USING ESSENTIAL OILS ON WATER QUALITY UNDER CONFINEMENT CONDITIONS

In a first factorial (3x3) test, the anaesthetic effect of the clove, mint and camphor essential oils were tested. Three different concentrations of each oil were tested simulating confinement conditions of transport, in periods of 6, 12 and 24 hours (n=8 fish per time/concentration). The clove, mint and camphor oils were evaluated at concentrations of 2.5, 5.0 and 7.5 µL L^{-1}; 20, 25 and 30 µL L^{-1}; and 100, 120 and 140 µL L^{-1}, respectively. These concentrations were determined based on a previous study (Pedrazzani e Ostrensky, 2014) and on a pilot test.

Essential oils were acquired from Ferquima Indústria e Comércio de Óleos Essenciais (São Paulo, Brazil). The camphor oil main compounds (percentage) were 35.5 1.8-cineole, 30.0 limonene, 13.0 alpha-pinene and 10.0 para-cymene, clove oil consisted of 85.0 eugenol and 13.0 beta caryophyllene. Mint oil was comprised of 37.0 l-menthol, 20.25 menthone, 6.75 limonene, 7.48 isomenthone, 4.60 menthyl acetate, 1.81 isopulegone, 1:39 pulegone, 0.08 carvone and 0.34 cineole. To obtain the respective concentrations of each anaesthetic, stock solutions were initially prepared by diluting each of the oils in 100% ethanol at a 1:10 ratio. All of the results were compared to those obtained from a control group in which the fish were subjected to the same procedure conditions but were exposed only to clean water.

To simulate confinement conditions comparable to those used for fish transport in Brazilian market, fish were randomly selected from the maintenance tanks and transferred to polyethylene bags measuring 16 x 30 cm at a density of 5 fish L^{-1}. The bags were then filled with 400 mL of saltwater and pure oxygen at a 1:2 ratio plus an anaesthetic at the respective experimental concentration. These containers were sealed with rubber bands and arranged in isothermal boxes identical to those used to transport ornamental fish, and remained there for the respective established period.

Physical-chemical parameters indicating water quality were measured immediately before the injection of oxygen and closing of the bags (initial values) and immediately after opening the packages (final values). The pH was measured using a benchtop digital pH metre (AZ 86505, Taiwan), and the concentration of dissolved oxygen and the water temperature were measured using a digital oximeter (YSI 550A, USA). The initial and final concentrations of NA-T were determined using the indophenol method (Apha, 2005a), followed by determination using spectrophotometry (Spectronic 20 Genesys, England). The concentration of gaseous ammonia ($N\text{-}NH_3$) was calculated using the formula proposed by Ostrensky *et al.* (1992) from the values for pH, temperature, salinity and N-TA concentration. The initial level of DO, temperature and pH before the experiment were 6.0 ± 0.18 $mg.L^{-1}$, 24.5 ± 0.32º C e 7.9 ± 0.01 (mean ± standard deviation), respectively. The concentrations of N-TA and consequently $N\text{-}NH_3$ remained below the detection limit of the method used.

After the confinement period, the fish were evaluated in terms of anaesthesia stage reached and were monitored for 72 h regarding mortality, presence of injuries and feeding behaviour. The considered stages of anaesthesia were I: Absence of reaction to touch and to visual stimulus; II: Initial loss of balance, characterized by difficulty to maintain normal swimming position III: Total loss of balance, uncoordinated swimming; IV: Minimal opercular movement, no swimming and V:

medullar collapse and no opercular beating, death. After, the animals were distributed evenly in a salt-water recirculation system with a 900 L capacity, divided into 15 storage tanks of 60 L. This system had similar environmental conditions to those in the maintenance tanks.

4.2.3 EFFECT OF FISH DENSITY ON WATER QUALITY USING ESSENTIAL OILS

A second experiment evaluated the effect of using clove, mint and camphor oils on water quality during confinement condition of *A. ocellaris* at different densities. For each oil, four densities of fish (5, 10, 15 and 20 fish L^{-1}) were tested with four replicates/treatment. The densities were determined based on the previous experiment and on commercial practices routinely adopted to transport clownfish in Brazil. To obtain that, five fish were placed in each bag (4 replicates/ treatment) and the volume of water containing anaesthetic was reduced according to the density increased. For the densities simulation of 5, 10, 15 and 20 fish L^{-1}, were added 1.000 mL, 500 mL, 333mL and 250 mL, respectively, of clean saltwater. The tested concentrations of clove, mint and camphor oils were 5, 25 and 120 µL L^{-1}, respectively. The results were compared with those obtained in a control group in which fish were exposed to the same conditions but without the addition of any substance to the water inside the bags.

The methods used for housing animals in polyethylene bags and determining the physic-chemical parameters of the transport water were similar to those described in section 4.2.2. In this experiment, the concentration of dissolved carbon dioxide (CO_2) in water was also measured by colorimetry and using sodium hydroxide and phenolphthalein (Apha, 2005b) before (initial) and after (final) a 24 h period. The initials levels of DO, CO_2, temperature and pH before the experiment were 5.89 ± 0.34 mg L^{-1}, 5.60 ± 0.49 mg L^{-1}, 24.5 ± 0.40º C and 7.9 ± 0.02 (mean ± standard deviation), respectively. The concentrations of N-TA and consequently N-NH_3 remained below the detection limit of the method used. Mortality and feeding behaviour were also evaluated during the 72 h immediately following the experiment.

4.2.4 STATISTICAL ANALYSIS

The normality of data was previously assessed by the Shapiro-Wilk test. Because the data did not fit a normal Gaussian distribution, analyses of significant differences between the tested variables were performed using the Kruskal-Wallis test ($p<0.05$). The results obtained using each anaesthetic at different concentrations, transportation times and densities were analysed separately. Subsequently, the outcomes of different anaesthetic treatments were compared in terms of transport water quality parameters. All analyses were performed using the software Statsoft Statistica™ version 10.0.

4.3 RESULTS

4.3.1 ANAESTHETIC EFFECT AND INFLUENCE OF USING ESSENTIAL OILS ON WATER QUALITY UNDER TRANSPORT CONDITIONS

No individuals died during the experiment. Animals subjected to the lowest concentration of clove oil (2.5 µ L^{-1}) showed no characteristic behavioural signs of anaesthesia. Animals exposed to 5 and 7.5 µL L^{-1} of this anaesthetic exhibited evidence of anaesthetic stage III and IV after 6 h of experimentation and stages II and III after 12 h. Mint oil concentrations of 20, 25 and 30 µL L^{-1} induced the animals to stages II, III and IV at 6 h, respectively. Fish exposed to the two highest concentrations of this same oil showed signs of stage II and IV anaesthesia after 12 h, respectively. The lowest concentration of camphor oil (100 µL L^{-1}) was sufficient to induce only stage II at 6 h, while 120 and 140 µL L^{-1} induced stage IV at 6 h and led to stages II and IV, respectively, after 12 h (TABLE 12). No behavioural signs of anaesthesia were identified in fish 24 h after starting the experiment at any tested concentration of clove and mint oils, in contrast to the results for the two highest concentrations of camphor oil, which were sufficient to keep the animals at anaesthetic stage II until the end of this period. Moreover, the highest concentrations tested for all oils caused the death of some animals, reaching 50% in the case of clove oil. The deaths always occurred between 12 and 24 h after the initial exposure to the anaesthetic.

TABLE 12. ANAESTHETIC STAGE AND MORTALITY (PERCENTAGE) OBSERVED AFTER TRANSPORTATION PERIODS OF *Amphiprion ocellaris* USING CLOVE, MINT AND CAMPHOR OILS AT THE RESPECTIVE CONCENTRATIONS IN ADDITION TO THE CONTROL TREATMENT.

Treatment	**Concentration ($\mu L\ L^{-1}$)**	**Stage**			**Mortality (%)**
		06h	**12h**	**24h**	
Control	0	—	—	—	0
Clove	2.5	—	—	—	0
	5.0	III	II	—	0
	7.5	IV	III	—	50.0 [a]
Mint	20	II	—	—	0
	25	III	II	—	0
	30	IV	IV	—	25.0 [a]
Camphor	100	II	—	—	0
	120	IV	II	II	0
	140	IV	IV	II	37.5 [a]

NOTE: [a] ALL DEATHS OCCURRED AFTER 24 HOURS OF TRANSPORT.

There were no significant effects on the water-quality variables of the three anaesthetics used considering different concentrations of the same anaesthetic, when comparing similar transport periods. However, there were significant differences in all of the tested water-quality parameters (TABLE 13 and TABLE 14) among the different periods studied.

TABLE 13. VALUES (MEDIAN, MINIMUM AND MAXIMUM) FOR DISSOLVED OXYGEN (DO) AND pH IN WATER AFTER DIFFERENT TRANSPORT PERIODS OF *Amphiprion ocellaris* USING THE CLOVE, MINT AND CAMPHOR OILS AND A CONTROL TREATMENT.

Treatament	Concentration ($\mu L\ L^{-1}$)	DO ($mg\ L^{-1}$)			pH		
		06h	12h	24h	6h	12h	24h
Control	0	17.75 [a] (12.3 – 18.9)	18.85 [b] (18.4 – 20.0)	19.20 [b] (18.3 – 20.0)	7.83 [a] (7.45 – 7.87)	7.60 [a] (7.50 – 7.60)	7.34 [b] (7.31 – 7.45)
Clove	2.5	14.30 (13.7 – 16.6)	13.45 (12.3 – 14.0)	13.80 (11.5 – 14.0)	7.92 [a] (7.91 – 7.92)	7.37 [ab] (7.24 – 7.63)	7.18 [b] (7.08 – 7.24)
	5.0	12.45 [a] (6.8 – 12.8)	11.70 [a] (10.8 – 12.4)	7.40 [b] (6.9 – 8.4)	7.91 [a] (7.89 – 7.93)	7.28 [ab] (7.26 – 7.30)	6.86 [b] (6.83 – 6.88)
	7.5	10.55 [a] (9.1 – 11.3)	11.75 [a] (10.9 – 12.2)	7.35 [b] (7.2 – 7.8)	7.91 [a] (7.86 – 7.94)	7.32 [ab] (7.28 – 7.41)	6.87 [b] (6.80 – 6.92)
Mint	20	16.30 [a] (15.8 – 16.8)	13.15 [b] (14.5 – 18.7)	15.10 [a] (14.1 – 16.9)	7.90 [a] (7.87 – 7.99)	7.13 [b] (7.07 – 7.21)	7.28 [ab] (7.27 – 7.32)
	25	15.20 [a] (13.4 – 16.2)	12.15 [a] (10.4 – 19.6)	11.75 [b] (11.2 – 12.9)	7.93 [a] (7.81 – 7.99)	7.24 [ab] (7.08 – 7.34)	6.97 [b] (6.93 – 6.98)
	30	14.20 [a] (13.4 – 15.2)	13.65 [a] (12.9 – 15.5)	11.40 [b] (10.2 – 12.2)	7.43 [a] (7.38 – 7.49)	7.21 [ab] (7.17 – 7.30)	6.99 [b] (6.98 – 7.00)
Camphor	100	17.65 [a] (16.6 – 19.3)	16.80 [ab] (14.5 – 18.7)	14.20 [b] (10.5 – 17.2)	7.81 [a] (7.53 – 7.87)	7.40 [ab] (7.34 – 7.43)	7.01 [b] (6.90 – 7.15)
	120	18.50 [a] (17.6 – 22.6)	17.05 [ab] (10.4 – 19.6)	14.35 [b] (13.6 – 17.4)	7.89 [a] (7.87 – 7.89)	7.39 [ab] (7.37 – 7.41)	7.11 [b] (7.07 – 7.16)
	140	18.55 [a] (18.0 – 19.1)	14.90 [b] (12.9 – 15.5)	16.00 [ab] (15.1 – 17.3)	7.88 [a] (7.87 – 7.98)	7.21 [b] (7.18 – 7.33)	7.17 [b] (7.11 – 7.20)

NOTE: DIFFERENT LETTERS INDICATE A SIGNIFICANT DIFFERENCE IN TRANSPORT TIMES (HORIZONTAL) ($p<0.05$). THERE WERE NO SIGNIFICANT DIFFERENCES IN THE VARIABLES AMONG DIFFERENT CONCENTRATIONS IN THE SAME TRANSPORT PERIOD (VERTICAL) ($p>0.05$).

TABLE 14. VALUES (MEDIAN, MINIMUM AND MAXIMUM) FOR CONCENTRATIONS OF TOTAL AMMONIA (N-TA) AND GASEOUS AMMONIA (N-NH_3) AFTER DIFFERENT TRANSPORT PERIODS OF *Amphiprion ocellaris* USING CLOVE, MINT AND CAMPHOR OILS AND NO ANAESTHETIC (CONTROL).

Treatment	Concentration (µL L^{-1})	N-TA (mg L^{-1})			N-NH_3 (mg L^{-1})		
		06h	12h	24h	06h	12h	24h
Control	0	0.73 ^{ab} (0.25 – 1.28)	0.50 ^{a} ((0.49 - 0.50)	1.20 ^{b} (0.30 – 1.90)	0.12 ^{ab} (0.04 – 0.16)	0.07 ^{a} (0.07)	0.13 ^{b} (0.06 – 0.23)
Clove	2.5	0.27 ^{a} (0 – 0.41)	0.00 ^{b} (0.00)	0.00 ^{b} (0.00)	0.05 ^{a} (0 – 0.08)	0.00 ^{b} (0.00)	0.00 ^{b} (0.00)
	5.0	0.22 ^{a} (0 – 0.45)	0.00 ^{b} (0.00)	0.00 ^{b} (0.00)	0.03 ^{a} (0 – 0.08)	0.00 ^{b} (0.00)	0.00 ^{b} (0.00)
	7.5	0.00 (0 – 0.13)	0.00 (0.00)	0.00 (0.00)	0.00 (0 – 0.02)	0.00 (0.00)	0.00 (0.00)
Mint	20	0.10 ^{ab} (0.09 – 0.10)	0.22 ^{a} (0.18 – 0.39)	0.00 ^{b} (0.00)	0.01 ^{a} (0.01)	0.01 ^{a} (0.01 – 0.03)	0.00 ^{a} (0.00)
	25	0.21 ^{a} (0.10 – 0.45)	0.21 ^{a} (0.18 – 0.43)	0.00 ^{b} (0.00)	0.03 ^{a} (0.01 – 0.08)	0.02 ^{a} (0.01 – 0.04)	0.00 ^{b} (0.00)
	30	0.24 ^{a} (0.10 – 0.65)	0.14 ^{a} (0.03 – 0.20)	0.00 ^{b} (0.00)	0.02 ^{a} (0.01 – 0.08)	0.01 ^{b} (0 – 0.02)	0.00 ^{b} (0.00)
Camphor	100	0.00 ^{a} (0.00)	0.11 ^{b} (0.01 – 0.23)	0.11 ^{b} (0 – 0.32)	0.00 (0.00)	0.01 (0 – 0.03)	0.01 (0 – 0.03)
	120	0.00 ^{a} (0.00)	0.20 ^{b} (0.01 – 0.34)	0.20 ^{b} (0.10 – 0.23)	0.00 ^{a} (0.00)	0.02 ^{b} (0.01 – 0.03)	0.02 ^{b} (0.01 – 0.02)
	140	0.00 ^{a} (0.00)	0.18 ^{b} (0.07 – 0.32)	0.00 ^{a} (0.00)	0.00 ^{a} (0.00)	0.02 ^{b} (0 – 0.04)	0.00 ^{a} (0.00)

NOTE: DIFFERENT LETTERS INDICATE A SIGNIFICANT DIFFERENCE IN TRANSPORT TIMES (HORIZONTAL) ($p<0.05$). THERE WERE NO SIGNIFICANT DIFFERENCES IN THE VARIABLES AMONG DIFFERENT CONCENTRATIONS IN THE SAME TRANSPORT PERIOD (VERTICAL) ($p>0.05$).

For all treatments, there was a declining trend in the DO concentration in water relative to the control over time. This same tendency is evident when comparing pH values, through water acidification occurred over the 24 h of experimentation in all treatments. At the beginning of the experiment, there were no detectable ammonia concentrations in the control. During the experiment, ammonia levels reached up to 1.90 mg L^{-1} of N-TA and 0.23 mg L^{-1} of N-NH_3 in the control. In treatments with camphor oil, there was an increase in the concentrations of N-TA and N-NH_3 after 12 h; however, these values remained virtually unchanged in the 24 h period. In contrast, for the treatments with clove and mint oils, except at a concentration of 7.5 µL L^{-1}, which remained stable, there was an increase in the concentrations of N-TA and N-NH_3 at 6 h and a decreasing trend between the periods of 12 and 24 h, when concentrations near zero.

4.3.2 POOLED ANALYSIS OF THE EFFECTS OF USING ANAESTHETICS ON WATER QUALITY

Since the data obtained of the variables (DO, pH, N-TA and N-NH_3) for different concentrations of each anaesthetic were not statistically different, it were analysed after pooling all of the experimentation periods, and some significant differences caused by the anaesthetics tested.

As seen in FIGURE 8, the use of anaesthetics caused DO concentrations in transport water to decrease by at least 10%, reaching 16.71 mg L^{-1} (measured in the camphor oil treatment). The lowest median value, 11.88 mg L^{-1}, was recorded in the clove oil treatment; this value was 56% higher in the control, reaching 18.57 mg L^{-1}. The treatments with anaesthetics featured greater variability in pH values (which ranged between 6.8 and 8.0) than the control (which ranged between 7.24 and 7.84). However, the greatest difference was observed in the concentrations of N-TA and N-NH_3. In this case, the medians measured for the control (0.61 and 0.08 mg/l of N-TA and N-NH_3, respectively) were significantly higher than those measured for treatments with clove (both medians were 0.00), mint (medians of 0.09 and 0.01) and camphor (medians of 0.02 and 0.00) oils.

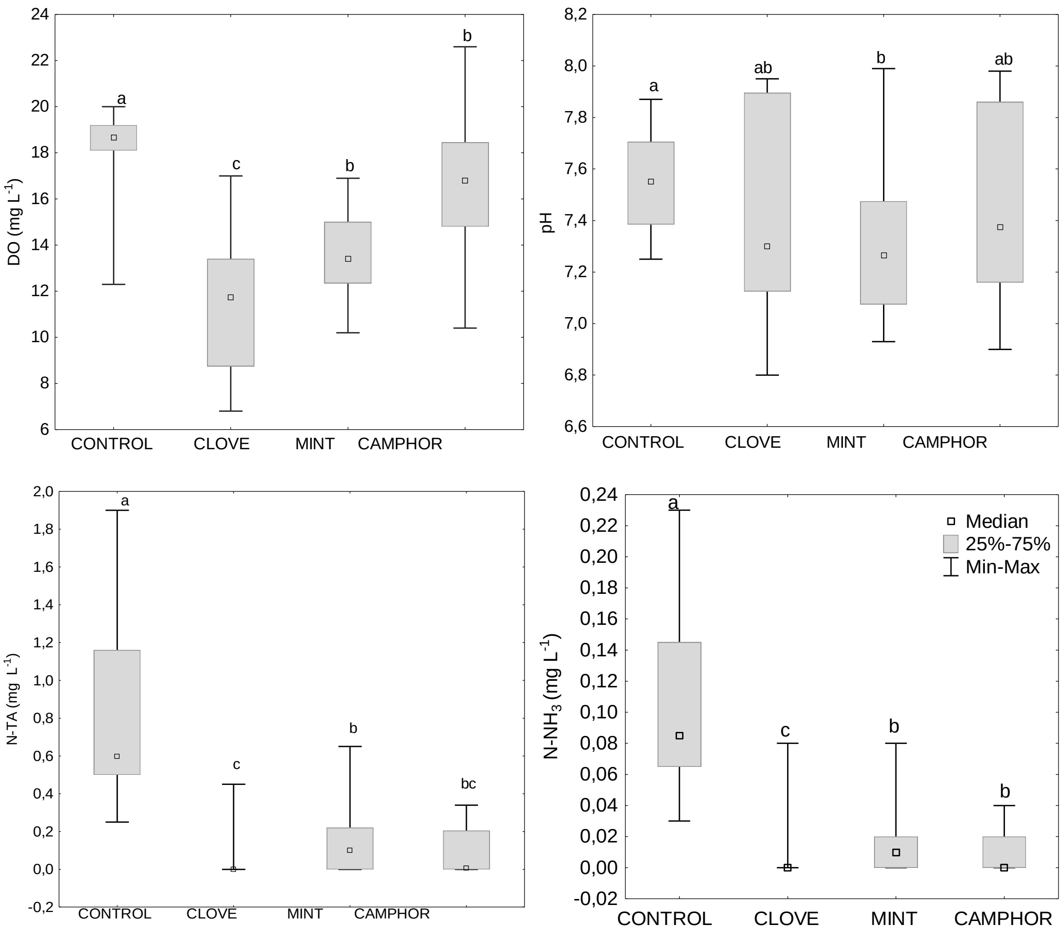


FIGURE 8. VARIATION OF CONCENTRATIONS OF DISSOLVED OXYGEN, pH, TOTAL AMMONIA AND GASEOUS AMMONIA IN TRANSPORT WATER USING CLOVE, MINT, CAMPHOR OILS AND NO ANAESTHETIC (CONTROL). DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES AMONG TREATMENTS ($p<0.05$).

4.3.3 EFFECTS OF DIFFERENT FISH DENSITIES ON WATER QUALITY DURING SIMULATED TRANSPORT CONDITIONS

Increasing the density of fish tended to increase the final measured concentrations of CO_2 and DO in practically all of the treatments and the control (Table 5). There was an opposite trend for pH: the values decreased with greater densities. The largest differences in median pH were measured in the control (0.27 pH units), while these differences ranged between 0.11 and 0.18 pH units for the other treatments. Concentrations of N-TA and N-NH_3 measured in the control and in the treatments containing clove and camphor oils showed a significant increase beyond the density of 10 fish L^{-1}. When using mint oil, such increase only occurred at densities starting from 15 fish L^{-1}.

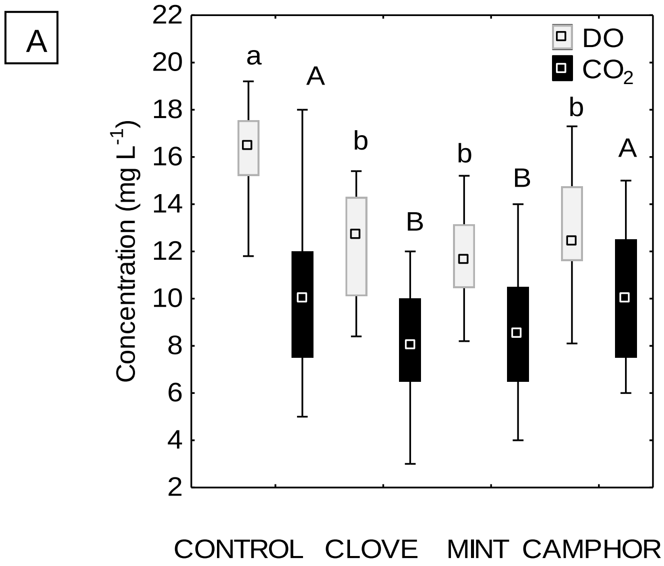
TABLE 15. CONCENTRATIONS (MEDIAN, MINIMUM AND MAXIMUM) OF DISSOLVED OXYGEN (DO), DISSOLVED CARBON DIOXIDE (CO2), TOTAL AMMONIA (N-TA), GASEOUS AMMONIA (N-NH3) AND pH OF WATER FOR 24 H AFTER THE BEGINNING OF EXPERIMENTS USING DIFFERENT DENSITIES OF *Amphiprion ocellaris* WITH CLOVE, MINT AND CAMPHOR OILS OR NO ANAESTHETIC (CONTROL).

Treatament	**Density Fish L^{-1}**	**DO ($mg\ L^{-1}$)**	**CO_2 ($mg\ L^{-1}$)**	**N – TA ($mg\ L^{-1}$)**	**N-NH_3 ($mg\ L^{-1}$)**	**pH**
Control	05	14.00 ^{a} (11.8 – 15.0)	05.0 ^{a} (5.0 – 8.0)	0.57 ^{a} (0.35 – 1.01)	0.06 ^{a} (0.03 – 0.10)	7.24 ^{a} (7.24 – 7.33)
	10	16.90 ^{b} (15.4- 17.8)	09.5 ^{b} (7.0 – 12.0)	2.59 ^{b} (2.15 – 2.73)	0.19 ^{b} (0.16 – 0.20)	6.97 ^{b} (6.93 – 6.98)
	15	17.10 ^{b} (16.0 – 17.6)	11.0 ^{bc} (10.0 – 12.0)	4.05 ^{c} (3.74 – 4.71)	0.31 ^{c} (0.30 – 0.36)	7.01 ^{c} (7.00 – 7.05)
	20	17.70 ^{b} (16.3 – 19.2)	13.5 ^{c} (11.0 – 18.0)	4.29 ^{c} (3.79 – 5.29)	0.36 ^{c} (0.29 – 0.39)	7.09 ^{c} (6.99 – 7.15)
Clove (5 µL L^{-1})	05	10.15 (9.4 – 13.3)	07.0 ^{a} (7.0 – 9.0)	0.00 ^{a} (0.00)	0.00 ^{a} (0.00)	6.97 ^{a} (6.83 – 6.99)
	10	12.95 (8.4 – 15.2)	08.0 ^{a} (6.0 – 9.0)	3.01 ^{b} (1.18 – 4.25)	0.22 ^{b} (0.08 – 0.31)	6.92 ^{a} (6.90 – 6.93)
	15	12.80 (9.1 – 15.4)	08.0 ^{ab} (6.0 – 12.0)	2.48 ^{b} (2.01 – 5.89)	0.17 ^{b} (0.13 – 0.40)	6.81 ^{b} (6.75 – 6.95)
	20	12.70 (10.4 – 14.3)	10.0 ^{b} (8.0 – 12.0)	3.72 ^{b} (3.49 – 4.70)	0.27 ^{b} (0.25 – 0.34)	6.86 ^{b} (6.84 – 6.87)
Mint (25 µL L^{-1})	05	10.90 ^{a} (9.2 – 12.6)	05.0 ^{a} (4.0 – 7.0)	0.29 ^{a} (0.03 – 0.43)	0.02 ^{a} (0 – 0.04)	7.13 ^{a} (7.11 – 7.16)
	10	09.55 ^{a} (8.2 – 12.1)	10.0 ^{bc} (8.0 -10.0)	0.28 ^{a} (0.19 – 0.45)	0.02 ^{a} (0.01 – 0.03)	6.95 ^{b} (6.92 – 6.96)
	15	13.40 ^{b} (10.9 – 15.2)	07.5 ^{b} (6.0 – 14.0)	2.61 ^{b} (2.22 – 3.25)	0.22 ^{b} (0.19 – 0.26)	7.05 ^{c} (7.02 – 7.10)
	20	13.15 ^{b} (11.2 – 15.2)	11.5 ^{c} (9.0 – 14.0)	3.03 ^{b} (2.21 – 3.17)	0.22 ^{b} (0.16 – 0.23)	6.97 ^{b} (6.94 – 6.98)
Camphor (120 µL L^{-1})	05	10.35 ^{a} (8.1 – 11.4)	07.0 ^{a} (6.0 – 8.0)	0.02 ^{a} (0 – 1.46)	0.00 ^{a} (0 – 0.09)	6.84 ^{a} (6.74 – 6.98)
	10	12.25 ^{b} (12.0 - 17.3)	09.0 ^{a} (7.0 -11.0)	1.16 ^{b} (0.42 – 1.98)	0.08 ^{b} (0.03 – 0.14)	6.98 ^{b} (6.91 – 7.03)
	15	13.10 ^{b} (11.8 – 14.8)	12.0 ^{b} (10.0 – 14.0)	1.88 ^{b} (1.28 – 3.24)	0.12 ^{b} (0.08 – 0.20)	6.86 ^{a} (6.81 – 6.87)
	20	14.80 ^{b} (12.6 – 15.6)	13.0 ^{b} (10.0 – 15.0)	1.89 ^{b} (0.04 – 2.35)	0.13 ^{b} (0 – 0.13)	6.73 ^{a} (6.73 – 6.75)

NOTE: DIFFERENT LETTERS INDICATE A SIGNIFICANT DIFFERENCE BETWEEN DENSITIES (VERTICAL) (p<0,05).

4.3.4 POOLED ANALYSIS OF DATA OBTAINED DURING THE SIMULATED TRANSPORT AT DIFFERENT DENSITIES

The final DO concentrations recorded in the control were 29 to 42% higher than those measured in treatments containing anaesthetics. In absolute terms, while the median DO in the control was 16.50 mg L^{-1}, the concentration for treatment containing mint oil was 11.65 mg L^{-1} (Figure 2). The CO_2 concentrations in the control and in camphor oil treatment were 10 mg L^{-1}, which is higher than the values in clove (8.0 mg L^{-1}) and mint (8.5 mg L^{-1}) oils treatments.



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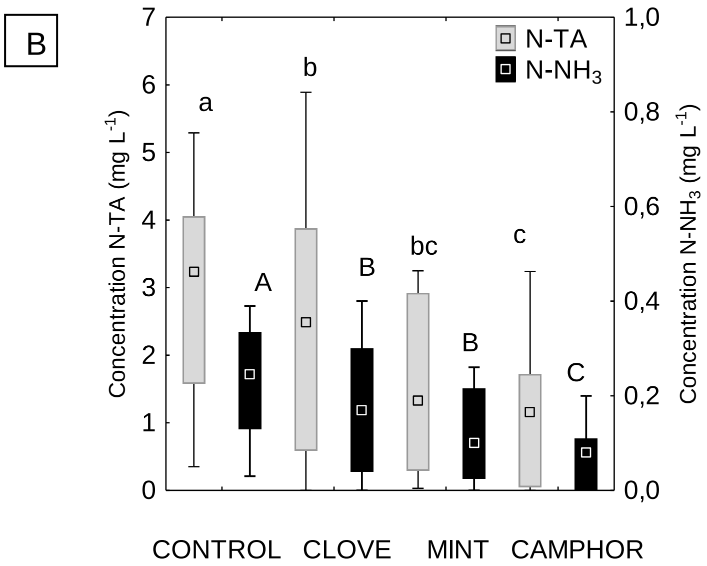

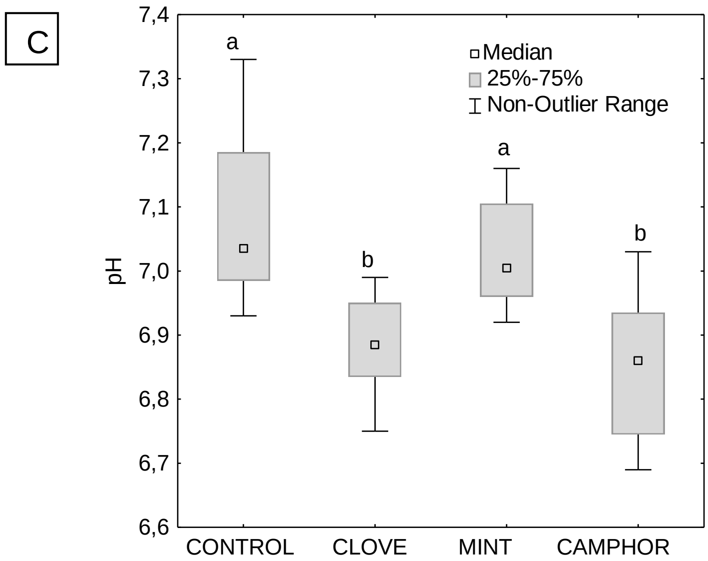

FIGURE 9. (A) RANGE OF CONCENTRATIONS OF DISSOLVED OXYGEN (DO) AND CARBON DIOXIDE (CO_2), (B) TOTAL AMMONIA (N-TA) AND GASEOUS AMMONIA (N-NH_3) AND (C) pH IN TRANSPORT WATER USING CLOVE, MINT AND CAMPHOR OILS OR NO ANAESTHETIC (CONTROL). DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES AMONG TREATMENTS ($p < 0.05$).

The median concentration of N-TA in the control was 3.23 mg L^{-1}, which was significantly higher than those observed in the treatments containing anaesthetic oils. A similar pattern was observed for N-NH_3, the concentration of which was 41, 140 and 200% higher in the control (median 0.24 mg L^{-1}) than the median concentrations measured in the treatments containing clove, mint and camphor oils, respectively.

The pH results can be split into two groups: one including the control (median 7.03) and the treatment with mint oil (median 7.00) and another group with lower values including the treatments with clove and camphor oils (medians of 6.88 and 6.86, respectively).

4.4 DISCUSSION

Establishing the concentration of a particular anaesthetic to be used during the transport of reef fish is very important but complicated. If the anaesthesia is too superficial, its effects can be null. However, if the anaesthesia is too deep and causes a total loss of balance, the animals can accumulate at the bottom of the container, which, according to Coyle *et al.* (2004), could lead to suffocation by overlapping fish at high densities. Death can also occur due to poisoning caused by long exposure to the anaesthetic itself because the safety margin decreases as the duration of exposure to the substance increases, thereby increasing the substance's toxicity (Marking, 1969; Ross e Ross, 2008). Advanced stages of anaesthesia involve reduced respiratory rate and efficiency, leading to a reduction in blood O_2 levels and a concomitant increase in CO_2 levels, which can lead animals to hypoxia (Thomas e Robertson, 1991). In most cases, maintaining stage IV anaesthesia for long periods without gill irrigation can result in death (Ackerman *et al.*, 2013). A combination of these factors could be responsible for the mortality that occurred in the treatments containing the highest concentrations of the anaesthetics tested in the present study.

Thus, among the concentrations tested, the intermediate ones (5, 25 and 120 $\mu L\ L^{-1}$ of clove, mint and camphor oils, respectively) are the most appropriate for use in transporting *A. ocellaris*. Although these concentrations resulted in a total loss of balance (stage III) during the first hours of transportation, they had no clear deleterious effects on the fish. At these concentrations, the animals remained in stage II anaesthesia (desirable) for most of the transport period. In other words, the fish were subjected to a relatively mild degree of anaesthesia that was still sufficient to safely mitigate the effects of adverse transport conditions.

The results showed a clear influence of transport time on the deterioration of water quality. In this regard, there were advantages and disadvantages of using anaesthetics. The major advantage concerns the tendency to reduce the ammonia level over time. In the absence of anaesthetics, animals excreted more and increasing ammonia concentrations in the water. Because there was less pH variation in the control, which remained at proportionately higher levels than in the treatments, especially in the cases of using clove and camphor oils, the concentrations of gaseous ammonia were significantly higher in the control than in treatments. Because gaseous ammonia is known to be toxic to fish, (Merkens e Downing, 1957; Ostrensky e Wasielesky Jr, 1995; Lim *et al.*, 2003) using anaesthetics can potentially reduce the risk of animal losses due accumulating concentrations of nitrogenous wastes during transport. In the control, $N\text{-}NH_3$ concentrations were recorded above 0.05 mg L^{-1}, which is considered the acceptable limit for marine fish (Cato & Brown, 2003), thus supporting this claim.

One disadvantage is the tendency for greater pH variability when using anaesthetics. However, the variation observed in the present study seems to have been insufficient to cause death or harm to the animals. According to Chow *et al.* (1994), *A. ocellaris* can withstand pH up to 6.3 under transport conditions, which is below the minimum value measured for all treatments. Mint oil allowed 10 fish L^{-1} to be transported without deleterious changes in water-quality parameters. In treatments containing clove and camphor oils, N-TA and $N\text{-}NH_3$ concentrations increased at this same density, indicating the lower efficacy of these oils at maintaining water quality at high densities. As expected, CO_2 increased and pH consequently decreased at higher densities. However, there was also an increase in DO concentrations in all treatments over the duration of the experiment. According to Pramod *et al.* (2010), elevated CO_2 concentrations reduce the capacity of haemoglobin to transport oxygen. Upon placing fish into an anaesthetic solution, large amounts of mucus are secreted into the water and can accumulate in the gills, thereby impairing gas exchange and consequently oxygen uptake, which was also reported by Chow *et al.* (1994) and Pandit e Ghosh (1999). Studies involving the use of anaesthetics during transport have also reported decreased oxygen uptake by guppy *Poecilia reticulata* (Teo e Chen, 1993), platy *Xiphophorus maculatus* (Guo *et al.*, 1995) and angelfish (Chellapan *et al.*, 2013). The DO concentrations measured after 6, 12 and 24 h were higher than the baseline for any of the treatments because pure oxygen was injected prior to closing the transport bags and the consumption was irrelevant compared to water oxygen absorption.

According to the data obtained in the present study, anaesthesia is suggested as a way of reducing the adverse effects of transport on *A. ocellaris.* Specifically, the results demonstrate the efficacy of using essential oils of mint at a concentration of 25 µL L^{-1} to transport a maximum density of 10 fish L^{-1}. At low densities (5 fish L^{-1}) clove and camphor oils at concentrations of 5 and 120 µL L^{-1}, respectively, can also be used safely to sedate fish during 24 h of confinement. It is also recommended to conduct studies on the use of buffering substances and ammonia removers in conjunction with essential oils during transport of *A. ocellaris.*

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APPENDIX I. USO DE ANESTÉSICOS DURANTE O MANEJO E O TRANSPORTE DO PEIXE-PALHAÇO *Amphiprion ocellaris*

Os peixes-palhaços *Amphiprion ocellaris* (FIGURA 1) são a espécie de peixe mais popular dos aquários marinhos residenciais. Parte dessa fama deve-se ao seu sucesso no filme “Procurando Nemo” e ao domínio das técnicas para seu cultivo em cativeiro. Porém, com o incremento da oferta de produtos e serviços voltados à piscicultura ornamental, houve também um natural aumento do nível de exigência dos consumidores por animais cada vez mais sadios; um acirramento da concorrência entre empresas por esse mercado; e, consequentemente, uma busca por aumento de eficiência, com necessidade de redução dos custos, para que os peixes-palhaços cheguem a preços cada vez mais atrativos aos consumidores.

Para que se produzam animais saudáveis e, ao mesmo tempo, com menor custo, são necessárias medidas de mitigação do estresse, que, por sua vez, geralmente está associado às práticas usuais de manejo, como a biometria e o transporte, feito usualmente em altas densidades. O uso de anestésicos pode ser ainda utilizado com o propósito de minimizar riscos de ferimentos durante a manipulação. Os peixes, quando anestesiados, consomem menos oxigênio e reduzem as taxas de excreção de resíduos metabólicos, como amônia e CO_2. Esses dois compostos são tóxicos e em condições normais de transporte podem atingir níveis que comprometem a sobrevivência dos animais. Por isso, peixes submetidos à anestesia correm menos riscos.

FIGURA 1. O PEIXE-PALHAÇO *Amphiprion ocellaris.*

A anestesia em peixes envolve cinco estágios, classificados de acordo com as alterações comportamentais que provocam, sendo I: perda de reação a estímulos externos; II: perda parcial de equilíbrio; III: perda total de equilíbrio; IV: redução dos batimentos operculares; e V: colapso medular e eventual morte. A recuperação anestésica ocorre quando o animal retorna o seu equilíbrio e passa a nadar normalmente de novo.

Durante procedimentos de rotina na aquicultura, como a manipulação para a biometria, o ideal é a obtenção do estágio anestésico IV, pois os animais, além de sedados, permanecem imóveis, facilitando o seu manuseio. Já para o transporte, o mais adequado é o estágio II, no qual há redução do metabolismo, sem haver perda total de equilíbrio, evitando assim, que os animais se acumulem no fundo da embalagem, o que poderia provocar o seu sufocamento.

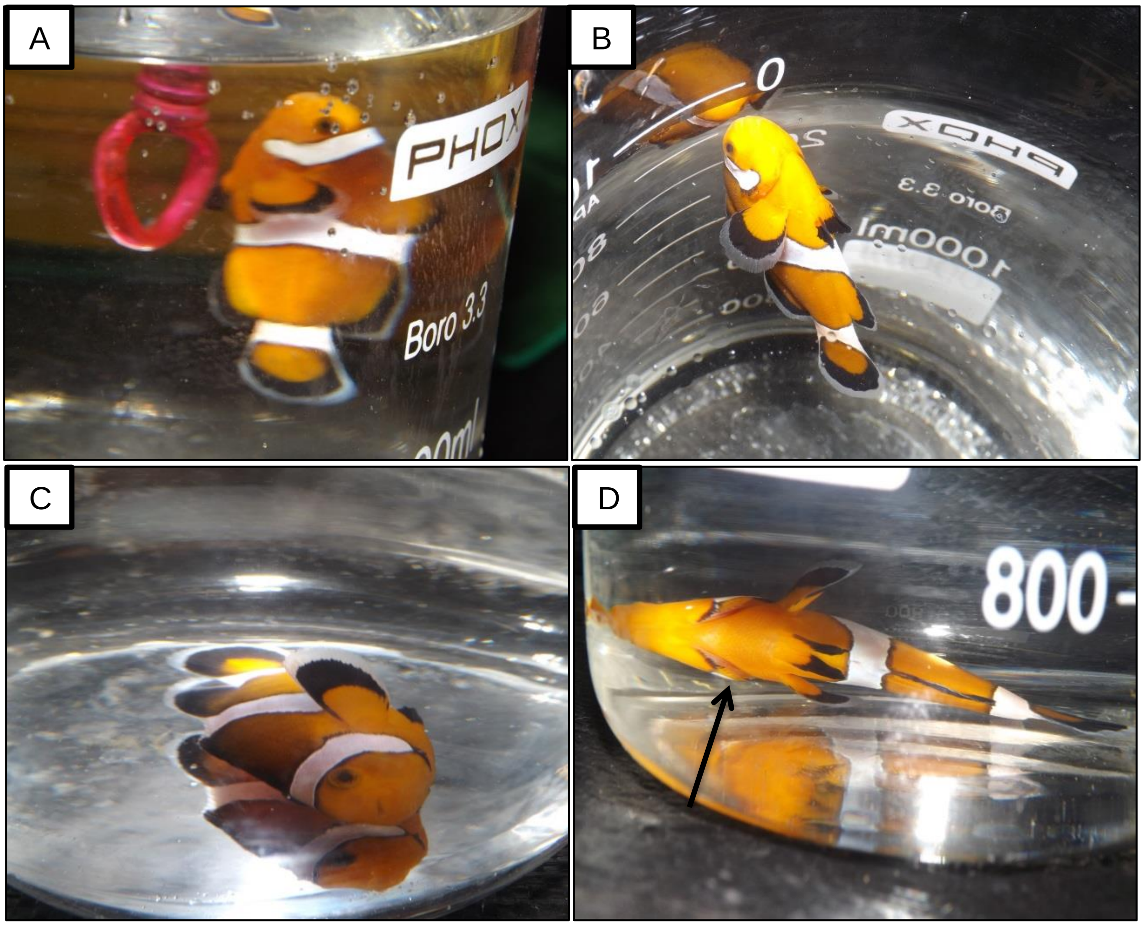

FIGURA 2. ESTÁGIOS ANESTÉSICOS EM *AMPHIPRION OCELLARIS:* (A) PERDA DE REAÇÃO A ESTÍMULO EXTERNO; (B) PERDA PARCIAL DE EQUILÍBRIO (C) PERDA TOTAL DE EQUILÍBRIO; (4) REDUÇÃO DE BATIMENTO OPERCULAR. NOTE OPÉRCULOS FECHADOS (SETA).

A escolha do anestésico a ser utilizado na piscicultura envolve o conhecimento da concentração ideal para a espécie em cada procedimento de manejo e os períodos necessários para indução e recuperação anestésicas. O desejável é que o anestésico proporcione obtenção a de estágio IV em, no máximo, 3 minutos (180 segundos) e o retorno da anestesia em 5 minutos (300 segundos).

O anestésico sintético utilizado com maior frequência na piscicultura é o MS-222 (tricaína metanossulfonato). No entanto, este composto tem venda controlada, e não é facilmente adquirido no cenário de produção, além de ter custo relativamente elevado e poder ser cancerinogênico para humanos e animais. O propofol (2,6 diisopropyl phenol) é um anestésico de baixo custo, mas pouco ainda se sabe sobre seus efeitos através de anestesia de imersão em peixes marinhos.

Experimentos realizados no Laboratório de Pesquisa em Organismos Aquáticos (LAPOA), do Grupo Integrado de Aquicultura e Estudos Ambientais (GIA), na Universidade Federal do Paraná (UFPR) para a comparação do desempenho anestésico do MS-222 e do propofol em *A. ocellaris* indicaram que os períodos necessários à indução anestésica por ambos anestésicos foram similares (cerca de 300 segundos), mas considerando a o período mais curto de recuperação anestésica, recomenda-se durante procedimentos de manejo, como biometria e classificação, a utilização de 80 mg L^{-1} de MS-222 (mais detalhes na tabela 1). Observou-se também que o propofol não proporcionou melhora na qualidade de água de transporte. Já o acréscimo de 15 mg L^{-1} de MS-222 na água de transporte, reduziu a eliminação de resíduos metabólicos durante a simulação de transporte da espécie em densidades de até 10 peixes L^{-1}, em período de 24 h. Porém, quando comparados os custos para sedação dos peixes, percebe-se que o uso e MS-222 pode ser um empecilho.

Os óleos essenciais extraídos de algumas plantas, como o cravo *Syzygium aromaticum* e a menta *Mentha sp*., podem substituir os anestésicos sintéticos, principalmente pelo baixo custo, pela facilidade de obtenção e pela segurança que proporcionam ao manipulador e ao meio ambiente. Ao avaliar o desempenho anestésico destes óleos para *A. ocellaris* foram obtidos tempos similares de anestesia em estágio IV entre eles (em torno de 300 segundos) e de recuperação anestésica (também em torno de 300 segundos). Também foi avaliado o uso de óleo de cânfora *Cinnamomum camphora* para anestesia desta espécie durante o manejo. Esse óleo proporciona uma indução mais lenta (535 segundos) e recuperação mais rápida (229 segundos) que os demais óleos essenciais. Quando utilizados em simulação de transporte, o uso de óleo de menta na concentração de 25 µL L^{-1}, na densidade máxima de 15 peixes L^{-1} promoveu redução significativa das concentrações de amônia na água, o que justifica o seu uso durante o transporte de *A. ocellaris*. Os óleos de cravo e cânfora nas concentrações de 5 µL L^{-1} e 120 µL $L^{-1,}$ também retardam os efeitos deletérios na qualidade da água, quando usado em baixas densidades de transporte (5 px L^{-1}). Estes compostos naturais, além de não proporcionarem riscos ao meio ambiente, demonstraram-se economicamente viáveis para uso durante o manejo e transporte do peixe-palhaço.

TABELA 1. CARACTERÍSTICAS DE DESEMPENHO DE ANESTÉSICOS UTILIZADOS PARA O PEIXE-PALHAÇO *Amphiprion ocellaris* em condições de manejo e de transporte.

	Anestésico	Concentração Manejo	Tempo (segundos) Indução	Recuperação	Custo/ 10L (R$)
Sintético	MS-222	80 mg L^{-1}	353,8	255,0	11,52
	Propofol	0,7 mg L^{-1}	280,0	1.507,5	0,85
Óleo Essencial	Cravo	27 µL L^{-1}	310,5	396,0	0,01
	Menta	70 µL L^{-1}	312,0	329,5	0,03
	Cânfora	500 µL L^{-1}	535,0	229,0	0,25

	Anestésico	Concentração Transporte	Duração (horas)	Densidade Máxima (px L^{-1})	Custo/ 10L (R$)
Sintético	MS-222	15 mg L^{-1}	24	15	2,16
	Propofol	0,3 mg L^{-1}	24	05	0,34
Óleo Essencial	Cravo	05 µL L^{-1}	12	05	<0,01
	Menta	25 µL L^{-1}	12	10	0,01
	Cânfora	120 µL L^{-1}	24	05	0,06

A análise desses resultados indica que, embora de formas distintas entre si, todos os compostos testados funcionaram como anestésico para o peixe palhaço. Por outro lado, todos eles, se utilizados de forma inadequada, podem colocar os animais em risco. Portanto, a decisão sobre qual anestésico utilizar passa obrigatoriamente pela experiência do manipulador, pela facilidade de obtenção do anestésico e pelo seu custo, pela margem de segurança proporcionada e pelo objetivo da anestesia.

APPENDIX II. ARTICLE RESULTED FROM SANDWICH DOCTORATE

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Reproductive behavior, embryonic and early larval development of the red head goby, *Elacatinus puncticulatus*

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ABSTRACT

The goals of this study are to provide a technical foundation for the production of the red head goby *Elacatinus puncticulatus* by evaluating its reproductive behavior and its embryonic and early larval development. Five pairs were kept under controlled conditions for thirty days. Courtship behavior, spawning period and the number of eggs produced were recorded. For the evaluation of embryo development, eggs were sampled at 12, 18, 24, 48, 72, 96, 120, 144 and 168 h post-fertilization(HPF). To test the influence of the incubation period on larval total length and height, eggs with six days (6D) of incubation and with seven days of incubation (7D) were subjected to flashlight illumination for 30 min to induce larval hatching. Another experiment evaluated the difference in larval survival with three different diets: *Euplotes* sp. (EU); rotifers *Brachionus rotundiformis* and *Brachionus plicatilis* and *Paramecium* sp. (BP); plankton collected from the wild (WP). The males displayed a gray head and pale yellow and black body coloration. Females exhibited strong red and black colors until three days before spawning, which occurred at intervals of 7 to 10 days. The hatching rate was 98–99%. The larvae total mean lengths and heights were 3.05 and 2.95 mm ($p > 0.05$) and 0.37 and 0.48 mm ($p < 0.05$) for treatments 6D and 7D, respectively. However, both groups exhibited high mortality at 5 days post-hatch (DPH). No larvae from the EU group survived after 5 DPH. At 8 DPH, 4% survivorship was found in treatment BP and 2% in treatment WP.

1. Introduction

The American Pet Products Association (APPA) revealed that 142 million freshwater fish and 9.6 million saltwater fish are kept for ornamental purposes in the United States (APPA, 2011). In recent years, there has been a large increase in the tendency to keep marine fish and corals in aquariums (Olivier, 2001). This increase has occurred because of the development of new technologies that facilitate the management of marine species in captivity and the decline in the price of these animals, which have become increasingly accessible to European and American markets. Most marine species marketed in the aquarium trade are collected in tropical and subtropical regions, particularly in coral reef areas, where the fauna displays a wide variety of colors and shapes. It is estimated that less than 10% of marine animals marketed for ornamental purposes originate from captive production (Wabnitz et al., 2003). The captive reared fish provide relief to the natural stock caused by fishery pressure and also offer other benefits of less aggressive behavior, the ability to readily feed on commercial dried feed, and reduced

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susceptibility to disease (Wittenrich, 2007).Currently, the major commercially cultivated species include the clownfish (*Amphiprion* sp.), gobies (*Gobiosoma* sp.), dottybacks (*Pseudochromis* sp.), seahorses (*Hippocampus* sp.) and Bangai cardinals (*Pterapogon kauderni*). Efforts are being made to develop technology for the breeding and rearing of at least 12 other species (UNEP, 2008).

The Gobiidae family consists of tropical species and represents 5% to 10% of all *Teleostei* species. They have great potential for the aquarium trade because of their bright colors, peaceful behaviors and ease of domestication and adaptation to commercial feed. The gobies of the genus *Elacatinus*, popularly called neon gobies, are small inhabitants of coral reefs. In addition to being ornamental, the neon gobies have the role of eliminating ectoparasites from larger fish and invertebrates (Gomes, 2010). Characteristics that favor their cultivation in captivity are the sex determination of *Elacatinus*, which is based on phenotypic characteristics and sexual behavior (Gomes, 2010), and the fact that they have demersal eggs and are considered prolific in captivity (Olivotto et al., 2011). However, there are few studies describing rearing protocols of *Elacatinus* sp., (Cortes, 2009; Meirelles et al., 2009; Shei et al., 2010; Souza, 2012).

The red head goby, *Elacatinus puncticulatus*, is a popular ornamental species because of the strong red and blue colors on the head and the yellow and black pigmentation of the body. This fish is native to the eastern side of the Pacific Ocean, occurring from North America to northeastern South America (Fishbase, 2012). Little information on the red head goby is available (Wittenrich, 2007) and therefore the goals of this study were to evaluate the reproductive behavior and embryonic and early larval development to provide a technical foundation for captive breeding of this species.

2. Materials and methods

This study was conducted through a partnership between the Laboratory of Research in Aquatic Organisms (LAPOA), Integrated Group of Aquaculture and Environmental Studies (GIA), Federal University of Paraná (UFPR), Curitiba, Paraná, Brazil, and the Vero Beach Marine Laboratory (VBML), Florida Institute of Technology, Vero Beach, Florida, USA. The study was conducted at the VBML.

2.1. Broodstock maintenance conditioning

Five pairs of *E. puncticulatus* formed six months before the start of the study were provided by Proaquatix®. Fish with a total length range between 3.5 and 5 cm were kept in individual 20 × 20 × 20 cm tanks interconnected to a recirculation system with a protein skimmer and biological filtration, a water heater and cooler. Two 4 cm long, 3 cm diameter PVC pipes were placed in each tank to provide a spawning habitat. Sand-filtered natural seawater from the Atlantic Ocean was maintained at a salinity of 33 g L^{-1} (±0.45), a temperature of 26 °C (±0.48), a pH of 7.9 (±0.04). The light intensity was maintained in 700 (±100 lx) and the photoperiod in 8L:16D. The levels of total ammonia, gaseous ammonia, nitrite and nitrate were maintained below 0.25 mg/L. Food (a mixture of shrimp, saltwater fish meat and micro-frozen and chopped crustaceans) was offered twice daily (8:30 and 15:30). One hour after each feeding, the bottoms of the tanks were siphoned to remove the excess food and feces.

2.2. Breeding, incubation and hatching

Observations of the reproductive behavior of red headed goby *E. puncticulatus* was observed three times a day (8:30 to 9:00, 11:30 to 12:00, 15:30 to 16:00) for thirty consecutive days (from September 17 to October 16, 2012) by the same observer. The courtship behavior, parental care and territorial behavior were recorded.

The spawned eggs were kept in the same aquarium as the broodstock so the male could perform cleaning and aeration until moments before hatching. Due to the difficulty of counting the eggs inside the PVC pipe, the total number of eggs was estimated. The PVC pipe was removed from the tank, while one of its exits remained closed to avoid water leak, and a flexible plastic ruler was introduced inside the pipe to measure the depth and length of the eggs mass. The total area of egg mass (TA) inside the PVC pipe was calculated by multiplying egg mass depth by the width. Then, the eggs were sampled (n = 10), and the individual area (IA) was measured using a 1 cm square grid under a dissecting microscope (10× magnification). To estimate the total number of eggs (NE), the area of egg mass (TA) was divided by IA (NE = TA/IA). Hatching occurred spontaneously and the hatching rate was estimated by counting the number of empty embryonic capsules after hatching. The incubation period of the eggs was recorded.

Embryo development was monitored using a dissecting microscope (10× magnification) and photographed regularly throughout the seven days of incubation. Three eggs per spawn from three different spawns were collected and sampled at 12, 18, 24, 48, 72, 96, 120, 144 and 168 h post-fertilization (HPF).

2.3. Effect of the incubation period on early larval development

The first experiment tested the influence of the incubation period on larval length and height. Different embryonic times were tested because of the significant loss of eggs on the last day of incubation and because it had been observed in the previous experiment that some larvae hatched before seven days of incubation. Eggs from two different pairs were pooled and tested after six days (6D) of incubation and, another group of larvae derived from the same two pairs but different clutches, after seven days of incubation (7D). They were subjected to flashlight illumination for 30 min to induce larval hatching. The newly hatched larvae from each treatment (n = 120) were randomly divided and transferred to three 20 L rectangular tanks (40 cm long × 25 cm wide × 20 cm deep) with the outside painted in black. The tanks were provided with frozen microalgae (*Nannochloropsis oculata*) and rotifers (*Brachionus rotundiformis*) previously enriched with commercial HUFA (Algamac®) as Olivotto et al. (2006). The physical–chemical water parameters were kept the same as those as in the broodstock tanks, and the photoperiod was maintained at 24L:0D. Low aeration was provided. Immediately after hatching, three larvae per replicate were euthanized with tricaine methanesulfonate (MS-222) at an overdose of 0.02 mg/L. Another three larvae were collected and sampled from both treatments at 24, 48, 72, 96 and 120 h post-hatch, placed in a Petri dish fitted with a 1 cm square grid and digitally photographed under a dissecting microscope (5× magnification).

2.4. Effect of different zooplankton diets on early larval survival

This experiment evaluated the differences in the survival rate of larvae fed three different diets. Larvae hatched after 7 days of incubation were used in this trial. The first treatment received only *Euplotes* sp. (EU), the second was fed with the rotifers *B. rotundiformis*, *B. plicatilis* and *Paramecium* sp. (BP) filtered between 50 and 300 screens, and the third received a diet based on zooplankton collected from the wild (WZ). Larvae from two different fish pairs were pooled and randomly divided into three different tanks, and stocked at a density of 50 larvae per tank. New and enriched batches of zooplankton were replaced twice daily in the larval tank. All the treatments were maintained at a density of 10 ind./mL, according to Olivotto et al. (2005). The necessary amount of zooplankton were harvested, washed with filtered seawater, and housed separately in 10 L plastic containers under aeration and light (1500 ± 100 lx) for enrichment. Zooplankton were enriched at the recommended rate of 0.5 g/L for 12 h (Bio-Marine, Inc., 2013).

To estimate the mortality, the larvae were counted on 0, 5, and 10 days post-hatch (DPH). One unique survivor larvae was sampled in the 17th day of life. To monitor growth, at the same stage of development, three larvae from each treatment were selected randomly, photographed and the total length and total height were measured. The gut contents of these samples were examined with an optical magnifier with 10× magnification to determine what had been eaten.

2.5. Plankton collection and cultivation

The rotifers *B. plicatilis* and *B. rotundiformis* were obtained from a sterile culture containing 100,000 individuals, which was kept in gentle aeration at 26 °C. A 25% water exchange was performed daily, and the rotifers were fed with *N. oculata* paste at a concentration of 50,000 cells/mL and added to the tank twice daily. Wild plankton was collected daily ($n = 10$), approximately 45 min after high tide, using a plankton net with a 50 μm mesh and a 50 cm diameter at the Sebastian Inlet, located in Sebastian, Florida, USA. The contents were sieved using 600, 120 and 50 μm mesh. The plankton between 50 and 120 μm in size were retained and used as food for the larvae. After each collection, the composition of the wild plankton was determined by sampling 1 mL of the collection in triplicate using an optical microscope lens at 10× magnification. The averages of each and of all collections were calculated. The wild-collected feed consisted of 85% copepods (*Parvocalanus crassirostris*) (larger diameter from 40 to 120 μm), 10% diatoms (*Coscinodiscus* sp.) and 5% dinoflagellates.

The *Euplotes* sp. and *Paramecium* sp. were isolated from the rotifer cultures through 25 and 50 μm filtration screens and cultured using gentle aeration and 25% water exchanges every three days. The *Euplotes* sp. culture was maintained at 50,000 cells/mL using algae paste (*N. oculata*).

2.6. Statistical analysis

The assumptions of parametric statistics (normality and homogeneity of variances, assessed by Shapiro–Wilk and Levene's tests) were met and therefore non-transformed raw data was used in all tests. A p value of 0.05 was taken for significance in all statistical tests. Differences between groups were analyzed in terms of the larval length and height using one-way analysis of variance followed by a multiple comparison Scheffé's method. Results were expressed as the means ± standard deviation (SD). The larval survival was analyzed by descriptive statistics (frequency). The statistical package used for analysis was Statistica Statsoft™ V. 10.0.

3. Results

3.1. Breeding, incubation and hatching

Of the five couples observed, three couples produced frequent spawnings, while the other two did not produce eggs. Spawning pairs also demonstrated active courtship, and parental care behaviors. During courtship and spawning, the males displayed a gray head and pale yellow and black body coloration. Females displayed strong red and black colors until three days before spawning and became pale in body coloration for the next three days prior to spawning. Two days prior to spawning, the female abdomen became swollen. One day prior to spawning, the female urogenital region changed from brown to red. Simultaneously, the males exhibited behavioral changes and promoted burrow cleanliness by moving their pectoral fins. During this period, the males displayed heavy breathing by increasing opercular movements and moving horizontally forward within the PVC pipe. Spawning occurred at intervals of 7 to 10 days approximately at 15:00 to 15:30. During the spawning, the couples remained side by side inside the PVC pipe. After spawning, the females were evicted from the PVC pipe by the males and usually remained in front of or above the PVC pipe. Females fed normally during the reproductive period except for the day of spawning during which time they stopped eating, while males did not feed during the incubation period but remained active providing aeration to the fertilized eggs.

The two couples that did not produce eggs did not display courtship behavior and occupied different PVC pipes throughout the observation period. Furthermore, no color differences were observed in non-reproductive pairs, which presented intense red head coloration and black lines in the body region.

Table 1
Number of spawning per pair, number of eggs spawned per clutch, with respective average and standard deviation.

Pair	Spawning				Mean	σ
	1º	2º	3º	4º		
A	225	240	100	175	185.00	63.11
B	150	125	120	160	138.75	19.31
C	45	180	180	–	135.00	77.94
Total					152.91	27.84

The number of spawned eggs was on average 152.91 ± 27.84 (Table 1), and the hatching rate was $98.5 \pm 0.547\%$. All males were observed cleaning the dead eggs. Two days before hatching, some spawns lost up to 50% of the eggs due to the cleaning maintenance performed by the males. The newly laid eggs were spherical in shape, ranging between 0.4 and 0.7 mm, with an average of 0.55 ± 0.11 mm in diameter.

At 12 HPF, the eggs displayed a translucent golden color. Within 12 HPF, the initial formation of the adhesive filament and the embryonic capsule was observed (Fig. 1A). At 18 HPF, the embryonic capsule was fully formed (with a height of 2.2 ± 0.2 mm and a width of 0.5 ± 0.1 mm), with four protuberances at the lateral distal region and one at the cranial egg region (Fig. 1B). At 24 HPF, the presence of a large yolk sac and early head formation were observed (Fig. 1C). At 48 HPF, detachment and growth of the caudal portion of the embryo in relation to the yolk sac was observed (Fig. 1D).

Within 72 HPF, there was a reversal in the position of the head relative to the filament adhesive, and the auditory placodes were present (Fig. 1E). At 96 HPF, the eggs turned transparent yellow, retinal pigmentation in the cranio-lateral portion of the embryo was observed, and the cranium to body ratio increased (Fig. 1F). Rapid growth of the embryos occurred at 120 HPF, at which time the majority of the capsular content was full, the circulatory system was formed and a heartbeat was observed (Fig. 1G). Furthermore, the anal pore, notochord and gas vesicle also became visible. At 144 HPF, the length of the tail was folded behind the head (Fig. 1H). Furthermore, melanization developed in the caudal portion of the embryo, and the yolk sac was also partially absorbed. The rudimentary formation of the mouth and intestine had also occurred. Pectoral fins and gill arches were also observed. At this stage, all eggs that were exposed to the microscope light for observation of embryonic development hatched. At 168 HPF, the embryos moved their tails intensely (Fig. 1I). When there was incidence of light, it was possible to observe the eyes moving, showing the presence of an ocular reflex. At this time there was also a decrease in yolk sac size, and spontaneous hatching of the larvae occurred. This process lasted approximately 1.5 h.

3.2. *Effect of the incubation period on early larval development*

The newly hatched larvae were transparent with some melanophores located near the caudal peduncle. The larval eyes were black with a prominent red metallic sheen when observed under the light. Larvae from treatment 6D displayed positive phototaxis, while those from treatment 7D did not. There was no significant difference in the mean total length of the newly hatched larvae, which were an average 3.05 ± 0.50 mm and 2.95 ± 0.35 mm for treatments 6D and 7D, respectively, (df = 19; $p = 0.960$; F ratio = 1.938; calculated $F = 0439$). In contrast, the average body height of the fish from the 7D incubation was 0.48 ± 0.06 mm, significant higher than the average body height of 0.37 ± 0.12 mm of those fish incubated for six days (df = 19; $p = 0.018$; F ratio = 4.217; calculated $F = 8.269$). Animals from treatment 6D displayed a closed or semi-open mouth (Fig. 2A), while larvae from treatment 7D had open mouths and a prominent jaw (Fig. 2B). A very small yolk sac was observed until 2 DPH. Full caudal fin development was present in both treatments. All sampled larvae of the 6D displayed a curved body at 5 DPH, complicating their movement and measurement (Fig. 2C). Larvae in treatment 7D presented greenish content in the intestine, while in 6D no content was observed; however, both groups experienced high mortalities at 5 DPH of larviculture.

3.3. *Effect of different zooplankton diets on early larval survival*

The larvae from all treatments showed a critical mortality period at 4 and 5 DPH. The survival in treatments EU, BP, and PA were 8%, 7%, and 12%, respectively, at day 5. No larvae in the EU group survived after 5 DPH. At 8 DPH, 4% survival was observed in treatment BP and 2% for treatment WP. By 18 DPH, one larva from treatment BP survived and was used for analysis (Fig. 2E). This last surviving larva showed slow development, with growth of 1 mm after 7 DPH and 1 mm after 18 DPH, when the total length was 5 mm. Live food densities decreased throughout the day for treatments BP and WP, yet food was only found in the digestive tract of larvae in treatment BP. In treatment EU, no decrease in food density was observed.

4. Discussion

The main challenges for the development of marine ornamental aquaculture are sexing and obtaining suitable broodstock and inducing spawning (Olivotto et al., 2011). Embryonic development is directly related to reproduction, broodstock maintenance conditions, incubation, and the transition from endogenous to exogenous feeding (Olivotto et al., 2011). Components that affect egg quality include the endocrine status of the female during the growth of the oocyte in the ovary, the diet of the broodfish and the complement of nutrients deposited into the oocyte (Brooks et al., 1997).

The incubation and the embryonic development of *E. puncticulatus* were very similar to *Elacatinus figaro* (Shei et al., 2010). In both species, the complete formation of the embryo occurs at 7 DPH, which highlights the importance of maintaining *E. puncticulatus* incubation for at least 7 days.

The stimulus for the larva to feed not only depends on the size of the food but also its color, shape and aroma (Olsen, 2007). The mouth size is directly related to the

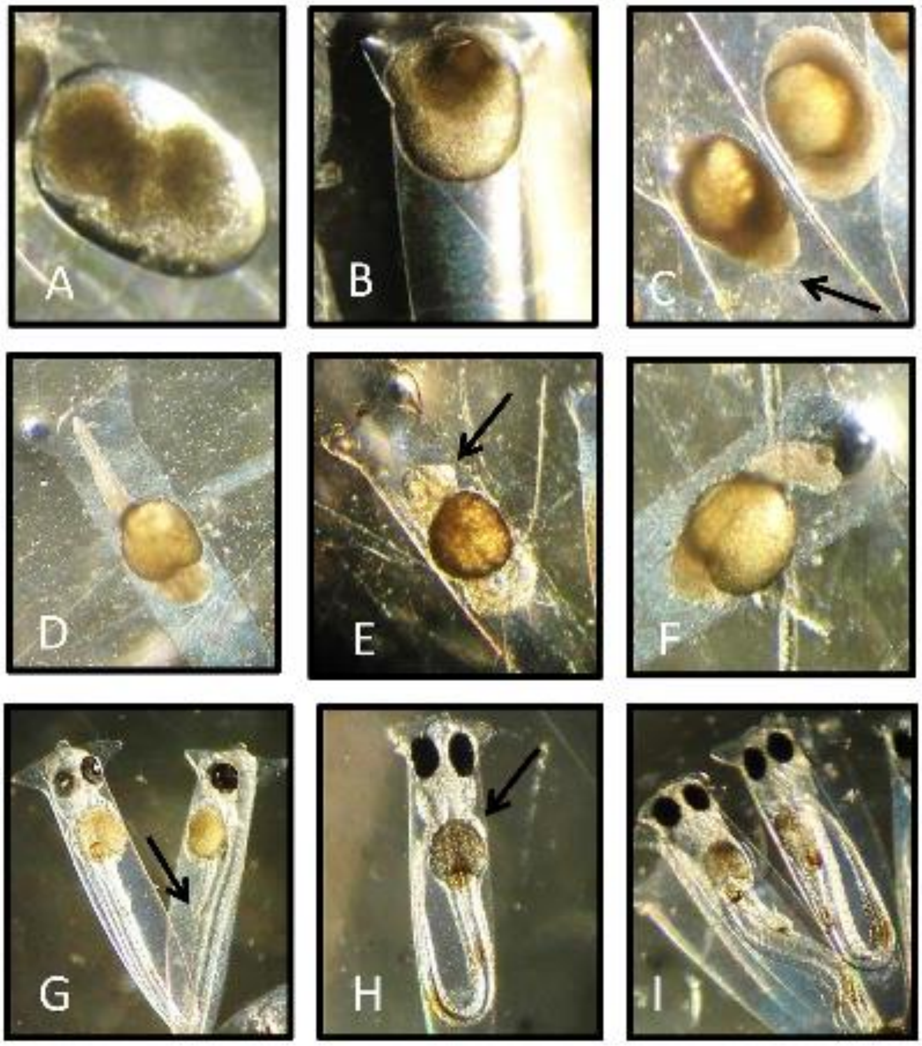

Fig. 1. Embryonic development of the red head goby, *Elacatinus puncticulatus*. (A) 12 HPF (hours post-fertilization), the early formation of the embryonic capsule (25× magnification); (B) 18 HPF, fully formed capsule and the germ ring reaching the vitelline pole (23× magnification); (C) 24 HPF, head constitution of the embryo (arrow) (22× magnification); (D) 48 HPF, significant body length increase (14× magnification); (E) 72 HPF, formation of the optical vesicle (arrow) (15× magnification); (F) 96 HPF, retinal pigmentation (26× magnification); (G) 120 HPF, anal pore formed (arrow) (10× magnification); (H) 144 HPF, development of pectoral fins (arrow) (15× magnification); (I) 168 HPF, larvae at hatching period (10× magnification). Note the empty embryonic capsule of newly hatched larva (left) and the strained tail of the larva in the center of the image (HPF—hours post-fertilization).

ability to catch food (Wittenrich, 2007). The biggest difference between treatments 6D and 7D was the formation of the mouth and jaw. The mouth opened immediately after hatching in treatment 7D larvae, at which point they could start to feed exogenously, which resulted in an increased survival. The period between the first feeding of the larva and the point at which larvae will inevitably die is fundamental to the mortality rate (Johnson and Katavic, 1986). The intense mobility observed in newly hatched 7D larvae suggests that they would have a greater ability to capture food and escape from predators (Ferreira et al., 2009). Copepods are small and fast swimming, which may have hindered successful food capture by the goby larvae. *Euplotes* sp. are spherical and very small (average of 100 μm of diameter) and also might not have been attractive to red head goby larvae. Despite the fact that the rotifer *Brachionus* sp. offers a low nutritional value to *E. figaro* (Souza, 2012) among the food items tested here, rotifers are an appropriate diet with regard to size, shape and ease of capture for *E. puncticulatus* larvae in its early stages of development.

In this study, enriching *Brachionus* sp. with commercial HUFA (Algamac®) was not enough to keep the animals properly nourished. According to Holt (2011), the levels of

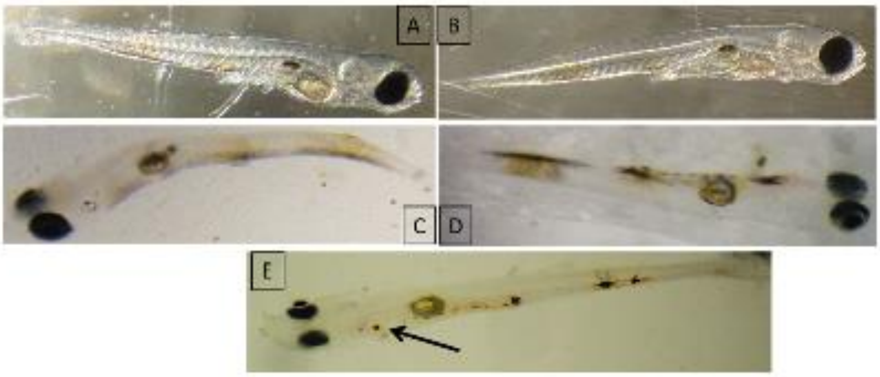

Fig. 2. Early larval development of the red head goby, *Elacatinus puncticulatus*. Note the closed mouth in newly hatched larvae in treatment 6D (A) (12× magnification) and the open mouth in treatment 7D (B) (10× magnification); (C) and (D) show the larvae at 5 DPH for treatments 6D and 7D, (9× and 7× magnification), respectively; (E) 18 DPH larvae (5× magnification). Note the presence of chromatophores on the dorsal portion of the larval body (arrow).

eicosapentaenoic (EPA, 20:5 $n-3$), docosahexaenoic (DHA, 22:6 $n-3$) and arachidonic (ARA, 20:4 $n-6$) fatty acids in the diet define the success of the physiological development of the nervous, sensory, genetic, immunological and homoeostatic balance systems. The high mortality that occurred at 5 DPH indicates that the larvae were not feeding properly. At 5 DPH, the yolk sac was completely absorbed, and no energy reserve was available for the larvae. The nutritional needs and food preferences of *E. puncticulatus* are critical because they feed naturally on parasites and cellular fish debris. The control of larval and broodstock nutrition will be essential for breeding this species in captivity. As no other studies on the species have been reported, these data may be used as a starting point for further studies to describe later larval development stages and cultivation of this and other species of the same genus.

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